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A rapid microextraction by packed sorbent - liquid chromatography tandem mass spectrometry method for the determination of dexamethasone disodium phosphate and dexamethasone in aqueous humor of patients with uveitis

Federica Bianchi[1*], Monica Mattarozzi[1], Nicolò Riboni[1], Paolo Mora[2], Stefano A. Gandolfi[2], Maria Careri[1]

[1]Dipartimento di Scienze Chimiche, della Vita e della Sostenibilità Ambientale, Università di Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy

[2] Unità di Oftalmologia - Dipartimento di Medicina e Chirurgia, Via Gramsci 14, Università di Parma, 43121 Parma, Italy

*Corresponding author:

Federica Bianchi: E-mail: federica.bianchi@unipr.it.

Phone: +39 0521 905446 ; Fax: +39 0521 905556

28 **Abstract**

29 A new method based on microextraction by packed sorbent (MEPS) coupled with liquid
30 chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated for the
31 determination of dexamethasone and dexamethasone disodium phosphate in human aqueous humor.
32 A central composite design was applied to investigate the effects of both loading and eluting cycles
33 in the MEPS procedure; subsequently the multicriteria method of the desirability functions allowed
34 to find the best conditions for the simultaneous extraction of both the analytes. Detection was
35 performed on a LTQ XL linear ion trap mass spectrometer operating in the positive electrospray
36 ionization mode applying multiple reaction monitoring mode. The assay was validated in
37 accordance with the guidelines bioanalytical method validation obtaining quantitation limits in the
38 low μg/L range, a precision characterized by RSD ≤ 16% and recovery rates in the 91 – 119%
39 range.

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47 **Keywords**: Microextraction by packed sorbent**;** Dexamethasone disodium phosphate;
48 Dexamethasone, Aqueous humor; Uveitis; Experimental design

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55 **1. Introduction**

56 Inflammatory disorders of the uveal tract of the eye (i.e. uveitis) are present all over the world with
57 an incidence in the developed countries ranging between 15 and 20 cases per 100,000
58 population/year. They may affect patients of any age, and represent a particular burden of disease
59 during early adulthood and between the age from 50 to 60 years [1]. Cataract formation is a
60 frequent complication of uveitis, as a sequel to inflammation itself or to the required steroidail
61 treatment. Cataract surgery in uveitic eyes has considerable potential for postoperative worsening or
62 relapsing of intraocular inflammation. Thus, perioperative dedicated prophylaxis may be warranted
63 [2]. In patients with a history of noninfectious uveitis (idiopathic or related to a systemic
64 autoimmune disease), the role of absolute control of inflammation continues with a greater focus on
65 perioperative supplementation with corticosteroids [3]. Among this class, dexamethasone –DEX-
66 (Fig. 1 left) is one of the most prescribed worldwide. It is a potent glucocorticoid (about 30 times
67 more than cortisone), used to treat a large variety of ocular diseases such as both anterior and
68 posterior segment inflammations, as well as to reduce inflammation following various ocular
69 surgeries [4]. In topical ophthalmic products, due to the low water solubility, dexamethasone is
70 usually replaced by the more hydrophilic dexamethasone disodium phosphate –DEX-SP- (Fig. 1
71 right) [5] that can be hydrolyzed to free desamethasone by phosphatase [6]. In order to obtain an
72 effective treatment of infection, dexamethasone has to reach the targeted site, achieving and
73 maintaining the therapeutic concentration of about 1 μg/mL [7]. The major drawback of the topical
74 administration of this drug lies in its poor permeability towards the corneal epithelium: in fact, less
75 than 3% of the instilled dose reaches the aqueous humor [8]. Dexamethasone concentration in the
76 vitreous humor is far lower when it is administered via eye drops rather than via
77 subconjunctival/subtenon injections. Only 0.001% of the drug when administered as an ophthalmic
78 solution is expected to reach the posterior segment, whereas about 0.01–0.1% is achieved through
79 periocular injections [8]. In addition, taking into account that dexamethasone's half-life is about 3-6
80 h [9], a frequent administration of the drug is required. Although these indisputable limits, topical

81 administration still remains ideally preferable. Systemic or intraocular steroids, indeed, pay a higher
82 availability with a greater risk for general and local side effects [10,11]. The analysis of
83 dexamethasone and dexamethasone sodium phosphate in aqueous humor requires high sensitivity
84 and selectivity due to the presence of these compounds at low concentration levels. Another
85 drawback lies in the low amount of biological sample that can be collected during surgery.
86 Gas chromatography-mass spectrometry (GC-MS) [12,13], liquid chromatography-mass
87 spectrometry (LC-MS) [13,14], liquid chromatography-tandem mass spectrometry (LC-MS/MS)
88 [13,15-17] and liquid chromatography-diode array detection (LC-DAD) [13,18] have been used to
89 analyze dexamethasone in different biological matrices, such as blood, urine and humor aqueous.
90 Compared to the GC-MS analysis, LC has the advantage of direct analysis of samples without a
91 derivatization step. In addition, LC-MS and LC-MS/MS were reported to provide lower detection
92 limits and better selectivity compared to LC-DAD analysis [13].
93 Various analytical methodologies, such as microextraction techniques, that reduce environmental
94 pollution, have been proposed for sample clean-up and analyte enrichment [13]. Among them,
95 microextration by packed sorbent (MEPS) proposed by Abdel-Rehi et al. in 2004, may represent an
96 interesting analytical option, since it combines sample processing, concentration and clean-up into a
97 fully-automated online sampling/injecting device [19, 20]. MEPS can be considered as a
98 miniaturized form of conventional solid phase extraction (SPE), by combining SPE phases inside a
99 special needle assembly that is then attached to the MEPS syringe. Compared to traditional SPE,
100 MEPS uses lower amount of sorbent. A noticeable advantage of this technique is the possibility to
101 process a wide range of sample volumes (from few μL to several mL) with high selectivity, thus
102 guaranteeing high-throughput analyses [21-23].As in the SPE, the core of the extraction is the
103 sorbent material: several commercial sorbents are available, being able to retain the analytes via
104 reversible interactions (hydrophilic, van der Waals, ionic, hydrogen bond, etc). The extraction
105 procedure consists of four main steps: i) sorbent conditioning; ii) sample collection; iii) sorbent
106 material washing; iv) analyte elution.

107 The present research study reports, for the first time, to the best of our knowledge, the development
108 and validation of a fast, efficient, sensitive, reliable and high throughput MEPS-based methodology
109 combined with LC-MS^2 for the simultaneous determination of dexamethasone sodium phosphate
110 and dexamethasone in human aqueous humor. Owing to the limited amount of sample available,
111 MEPS proved to be the technique of choice to perform extraction, clean-up and pre-concentration
112 of the investigated analytes in one step.

113

114 **2. Materials and methods**

115

116 *2.1. Chemicals and materials*

117 Dexamethasone-21 disodium phosphate salt (> 98% purity), dexamethasone (≥ 98% purity), acetic
118 acid (99% purity), ammonium acetate (98% purity) and methanol (>99.9 % purity) were purchased
119 from Sigma-Aldrich (Milan, Italy).
120 C2, C8 and C18 MEPS BIN were from SGE Analytical Science (Milton Keynes, UK).

121

122 *2.2. Aqueous humor sampling*

123 Following respective ethics committee approval, patients with a history of uveitis requiring cataract
124 surgery were divided into two groups of perioperative steroidal supplementation. Group A (topical
125 prophylaxis alone): from 4 days before surgery: disodium dexamethasone 0.15% eye drops (4
126 instillations/day). Group B (topical + oral prophylaxis): from 4 days before surgery: oral
127 dexamethasone (0.05 mg/Kg/day, in a single dose in the morning), and adjunctive topical treatment
128 as in the group A, as detailed in a previous report [24]. A total of 15 patients per group were
129 involved. The day of surgery the patients were asked to put one drop of dexamethasone 0.15% in
130 the operating eye just before moving to the Hospital (2 to 4 hours before surgery). Cataract
131 extraction was performed on all patients through standard pharmacoemulsification technique with
132 foldable hydrophobic acrylic lens implantation, under topical or local anaesthesia (peribulbar

133 injection). After cutting the corneal limbus, before the filling of the anterior chamber of the eye with
134 viscoelastic matrix, an aliquot part of the aqueous humour (at least 50 μL, depending on the
135 anatomy and the clinical characteristics of the eye) was drawn with a suitable sterile syringe with
136 flat needle. This withdrawal did not change the modalities and the prognosis of surgery, being the
137 aqueous humor normally dispersed on the surgical field. The liquid was quickly transferred in a
138 microcentrifuge tube and stored at -80°C. From subjects who agreed to enter the study, written
139 informed consent was obtained according to the tenets of the Declaration of Helsinki.
140
141 *2.3. Experimental design and optimization of the MEPS procedure*
142 The experiments were carried out on blank aqueous humor samples spiked with DEX-SP and DEX
143 both at 3 μg/L.
144 A 2^2 two-levels full factorial design (FFD) [25] was carried out by investigating the effects of both
145 loading and eluting cycles. In both cases low and high levels were 5 and 25 cycles, respectively. A
146 *F*-test comparing the experimental and calculated responses at the centre of the experimental
147 domain was performed to evaluate the existence of relevant quadratic effects and a star design was
148 added to the factorial design experiments [26]. The final regression models were then calculated
149 using the Central Composite Design (CCD) experiments, obtained both from the FFD and the star
150 design and used to find the optimal extraction conditions by using the multicriteria method of the
151 desirability functions [27-29]. All statistical analyses were carried out by using the statistical
152 package SPSS Statistics 24.0 (IBM, Milano, Italy).
153
154 *2.4. MEPS procedure*
155 A commercial e-Vol® equipped with a C18 Barrel Insert and Needle (BIN) was used for MEPS
156 extraction. Prior to extraction, each BIN was activated by using 4x50 μL of methanol and 2x50 μL
157 of water. Fifty μL of aqueous humor were drawn up and down through the BIN 19 times. Then, the
158 analytes were eluted with 10x26 μL of methanol and analyzed by LC–MS^2. After extraction, 10

159 wash cycles each with 10x50 µL of methanol were used to clean the sorbent material and to avoid
160 carryover effects. Both a fill and injection speed of 1 arbitrary unit were used.
161
162 *2.5 LC-MS^2 analysis*
163 Chromatographic separation was performed on a HPLC system (Thermo Electron Corporation, San
164 Josè, CA, USA) coupled with a LTQ XL linear ion trap mass spectrometer (Thermo Electron
165 Corporation) equipped with a pneumatically assisted electrospray (ESI) interface. The system was
166 controlled by the Xcalibur software (Thermo Electron Corporation). The mobile phase was
167 delivered by the Surveyor chromatographic system (Thermo Electron Corporation) equipped with a
168 200-vial capacity sample tray. A volume of 20 µL of each extract was injected into a GEMINI C18
169 100 mm x 2.0 mm, 3 µm 110 Å column (Phenomenex, Torrance, California, USA), equipped with
170 a C18 security guard cartridge and thermostated at 25 °C, at a flow rate of 250 µL/min. The mobile
171 phase consisted of solvent A (methanol) and solvent B (5 mM ammonium acetate) (pH=4.2). The
172 initial condition was 40% solvent A and 60% solvent B. A linear gradient was performed with
173 mobile phase A increasing from 40 to 90% within 1 min. After 7 min the mobile phase was returned
174 to the initial conditions and re-equilibrated for 2 minutes. The sheath gas (nitrogen, 99.99% purity),
175 the auxiliary gas (nitrogen, 99.99% purity) and the sweep gas (nitrogen, 99.99% purity) were
176 delivered at flow rates of 30, 10 and 5 arbitrary units, respectively. Optimized conditions of the
177 source were set as follows: ESI voltage, 4.5 kV; capillary voltage, 30 V; capillary temperature,
178 300°C; tube lens, 100 V. DEX-SP and DEX were analysed in multiple reaction monitoring (MRM)
179 mode using an electrospray probe in the positive ionization mode. The following transitions were
180 monitored: *m/z* 517 → *m/z* 499 (used for quantitation), 479 and 395 for DEX-SP and m/z 393 →
181 373 (used for quantitation), 355 and 337 for DEX. A collision energy of 30 V was used.
182
183
184

185 *2.6 Validation*

186 Validation was carried out under the optimized conditions, to meet the acceptance criteria for
187 bioanalytical method validation [30].

188 Aqueous humor samples extracted from patients submitted to cataract surgery not treated with
189 DEX-SP were used as blank matrix. For both the analytes, the lower limit of quantification (LLOQ)
190 was calculated as signal-to-noise ratio, S/N = 5, using five independent samples and tested for
191 accuracy and precision to meet the previously cited international criteria. The calibration curves
192 were evaluated on six concentration levels in the LLOQ-150 µg/L range, performing two replicated
193 measurements for each level. Lack-of-fit and Mandel's fitting test were performed to check the
194 goodness of fit and linearity. The significance of the intercept (significance level 5%) was
195 established by running a Student *t*-test. Precision in terms of both within-run and between-run
196 precision was calculated in terms of RSD % on three concentration levels (LLOQ, 3 and 70 µg/L),
197 performing five replicates at each level. Between-run precision was estimated over three days
198 testing for homoscedasticity among the data and performing the analysis of variance (ANOVA) at
199 the confidence level of 95%. Accuracy was calculated in terms of recovery rate (RR%) as follows:

200 $RR\% = c_1/c_2 \times 100$

201 where c_1 is the measured concentration and c_2 is the concentration calculated from the quantity
202 spiked into the sample. Three different concentration levels (LLOQ, 3 and 70 µg/L) with five
203 replicated measurements were analyzed. The extraction yields in terms of percent recovery was
204 calculated by comparing the results obtained from the MEPS-LC-MS^2 analysis of the biological
205 matrix spiked both with DEX-SP and DEX at three concentration levels (LLOQ, 3 and 70 µg/L)
206 with those obtained for the true concentration of the analyte in solvent (n=5).

207 Method selectivity was assessed by testing for interference 10 blank aqueous humor samples.

208 Finally, stability was evaluated in terms of both long-term and bench-top storage, and processed
209 sample stability. Three replicates at the LLOQ and at 70 µg/L were always performed.

210

211 **3. Results and discussion**

212

213 *3.1.MEPS optimization*

214 Taking into account that the aim of the study was the quantitation of DEX-SP and DEX in the
215 aqueous humor of patients affected by uveitis requiring cataract surgery in order to evaluate the
216 effectiveness of the topic administration of the corticosteroids,the microextraction by packed
217 sorbent approach is very attractive in bioanalysis to promote both the purification of the matrix and
218 the concentration of the analytes, since reduced size of sample is required. According to previous
219 published studies [18,31], preliminary investigations carried out by using C2, C8 and C18 BINs,
220 showed that the highest extraction yields could be obtained by using C18 and methanol as packed
221 sorbent and eluting solvent, respectively (Figg. 2,3). In order to optimize the MEPS conditions, a 2^2
222 FFD was performed by investigating the effects of both loading and eluting cycles. The
223 experimental domain was defined taking into account that in order to favor a good interaction
224 among the analytes and the sorbent material, a minimum number of loading cycles have to be
225 performed. In our study this value was set as n=5. The same was for the elution process. As for the
226 fill/injection speed, a value of 1 arbitrary unit was used to avoid the presence of bubbles in both the
227 filled and in the eluted solutions. Repeatability of measurements was assessed by performing 4
228 replicates at the centre of the experimental domain. For each compound, main and interaction
229 effects were calculated. The presence of curvature was assessed for both the analytes, thus requiring
230 to perform the experiments corresponding to a star design. Table 1 list the regression models used
231 to search for the highest MEPS-LC-MS^2 response by means of the multicriteria method of
232 desirability functions. The optimal experimental conditions were found in correspondence to a
233 number of loading cycles=19. Taking into account that a global desirability D=0.90 and that very
234 good single desirability values were obtained, the developed procedure proved to be suitable for the
235 simultaneous extraction of the investigated corticosteroids. As for the eluting cycles, taking into
236 account that samples containing higher amounts of corticosteroids compared to the 3 μg/L used in

237 the CCD could be analyzed, a number of cycles n=10 was used to favor the complete elution of the
238 analytes.
239

240 *3.2 Method validation*

241 The method was validated using the experimental setting providing the optimized conditions (Table
242 2). LLOQ values of 0.7 and 0.5 µg/L for DEX-SP and DEX, respectively were calculated, thus
243 attesting the capability of the developed method of quantifying the analytes at trace levels.
244 Good linearity was proved by applying Mandel's fitting test in the LLOQ-150 µg/L range for both
245 the analytes. Satisfactory precision was proved both in terms of within-run and between-run
246 precision with RSD always lower than 16% also at the LLOQ levels, thus meeting the criteria as
247 described in the guidelines for the validation of bioanalytical methods [30].
248 Recoveries in the 91(±6)–119(±25)% (n=5) range proved the accuracy of the developed method,
249 whereas extraction yields higher than 85% were always obtained.
250 The method has shown good selectivity, since there was no interference from other endogenous
251 compounds . As for stability, no problems related to the long-term stability of the stock solutions
252 were observed: in fact, ANOVA performed on data obtained by the analysis of standard solutions
253 daily prepared from the stock solutions did not show significant differences ($p>0.05$) up to 7
254 months when the stock solutions were stored at -20°C. Bench-top stability was evaluated by
255 analyzing standard solutions maintained at room temperature up to 12 h at two concentration levels.
256 By applying the student t-test, no significant differences ($p>0.05$) were observed between the mean
257 responses. Freeze and thaw stability was not assessed, since it was not possible to perform freeze
258 and thaw cycles due to the low volume of available sample.
259 Finally, the processed sample stability proved that no significant differences between the responses
260 obtained from the same sample analyzed just after the MEPS extraction and after 10 h. This time
261 was the longest period until completion of the analysis.
262

263 *3.3 Real sample analysis*

264 In order to investigate the validity of the devised method, aqueous humor real samples were
265 analyzed as described in 2.2. Section. Neither DEX-SP nor DEX were detected in samples from
266 group A and B, respectively. The obtained results can be explained taking into account both the low
267 number of patients and the time elapsed from the drop instillation. Although one study reported that
268 DEX could be detected 12 h after instillation [12], it is known that along the time a drastic decrease
269 in the concentration of the analytes occurs [5, 31-33]. These evidences reinforce the interest in
270 newly developed ophthalmic drug carries for topical administration, as cyclodextrin-poloxamer
271 aggregates; they also offer to such nanocarrier topical administration a suitable method to assess
272 intraocular concentrations [34-36].

273

274 **4. Conclusion**

275 A rapid and reliable MEPS-LC-MS^2 method for the determination of dexamethasone disodium
276 phosphate and dexamethasone in humor aqueous was developed and validated. Multi-criteria
277 optimization method based on the desirability function approach allowed for the simultaneous
278 determination of both the analytes at trace levels. The proposed method allowed for rapid analysis
279 time, low volume consumption, good selectivity and high extraction yield, thus being a valuable
280 tool for clinical studies to assess reliability of dexamethasone administration in patients affected by
281 uveitis.

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289 **5. References**

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390 **Figure captions**

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392 **Fig. 1.** Molecular structure of dexamethasone (left) and dexamethasone disodium phosphate (right)

393 **Fig. 2.** MEPS sorbent selection. Three replicate measurements for each sorbent

394 **Fig. 3.** MEPS eluting solvent. Three replicate measurements for each solvent

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