Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 805 (2004) 255–260
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Separation and determination of dexamethasone sodium phosphate in cochlear perilymph fluid by liquid chromatography with ultraviolet monitoring and electrospray ionization mass spectrometry characterization

Hongxia Liu [a,*], Xiaolan Chen [a], Shusheng Zhang [a], Lingbo Qu [a], Yufen Zhao [a,b,1], Hongjian Liu [c], Mingmin Dong [c]

[a] *Department of Chemistry, The Key Laboratory of Chemical Biology and Organic Chemistry of Henan, Zhengzhou University, No. 75 Daxue Road, Zhengzhou 450052, PR China*
[b] *Department of Chemistry, The Key Laboratory for Bioorganic Phosphorus Chemistry, Tsinghua University, Beijing 10084, China*
[c] *The First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, PR China*

Received 15 October 2003; received in revised form 28 February 2004; accepted 4 March 2004

Available online 15 April 2004

Abstract

The method for separation and determination of dexamethasone sodium phosphate (DexP) in cochlear perilymph fluid (CPF) of cavy was developed using HPLC with ultraviolet (UV) monitoring and electrospray ionization/mass spectrometry (ESI/MS) identification. The quantitative determination of DexP in CPF was achieved by HPLC with UV detection at 245 nm. The separation was carried out on a Phenomenex ODS(3) column (250 mm × 4.6 mm i.d., 5 μm) with the mobile phase of acetonitrile–5 mmol/l ammonium acetate (23:77 (v/v)) at a flow rate of 1.0 ml/min. DexP was baseline separated from the matrices of CPF blanks within 15 min. The linearity ranged from 0.5 to 50 μg/ml. The limit of detection was 0.10 μg/ml. The recovery ranged from 98.5 to 100.8%. The relative standard deviations (R.S.D.s) of intra- and inter-day peak area were between 0.7–1.3 and 1.2–3.5%, respectively. Both full scan MS and MS^2 of DexP with positive and negative polarity were obtained and elucidated. The specific ions were chosen to characterize DexP in the CPF sample. Using the proposed HPLC-UV-ESI/MS method, the concentration of DexP in CPF samples after both vein and middle ear injections were determined, and the relationships between concentration and time were obtained. This method offered reference data for clinical investigation of DexP to cure ear diseases.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Dexamethasone sodium phosphate

1. Introduction

Dexamethasone sodium phosphate (DexP), an analog of corticosteroid hormones, has been widely used to treat inflammation, allergy and diseases related to adrenal cortex insufficiency. Currently, the clinical observations have shown that DexP features excellent curative effects in treating ear diseases [1–4]. However, it has been demonstrated that the pharmaceutical effects of DexP strongly depended on the administered track and distribution of DexP in tissues and body fluids. For a better understanding of these effects, a detailed investigation on the pharmacokinetics of DexP is very necessary. To date, Sun et al. [2] have reported an investigation on the pharmacokinetics of DexP in endolymph fluid of cavy. However, to the best of our knowledge, the investigation on its pharmacokinetics in cochlear perilymph fluid (CPF) of cavy has so far not been documented.

Several approaches have been employed to measure DexP in body fluids, e.g. spectrophotometry [5,6], polarimetry [7], capillary electrophoresis [8,9], thin layer chromatography [10] and high performance liquid chromatography (HPLC) [11,12]. Hitherto, HPLC has proven to be the most prevalent technique for assaying preparation, dosage and biological samples [1,11–13]. In the work described in this paper, a practical and reliable HPLC, based on

* Corresponding author. Tel.: +86-371-7763224; fax: +86-371-7763224.
E-mail address: liuhongxia999@371.net (H. Liu).
[1] Also corresponding author. Tel.: +86-371-7767050.

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.03.005

UV detection and electrospray ionization/mass spectrometry (ESI/MS), has been developed for identification and determination of DexP in CPF of cavy. Meanwhile, the HPLC-UV-ESI/MS method has been successfully applied to achieve the concentration–time curves of DexP in CPF.

2. Experimental

2.1. Chemicals and solutions

Unless specified otherwise, all chemicals and solvents were of analytical reagent grade and were obtained from Beijing Chemical Factory (Beijing, China). Methanol and acetonitrile were HPLC grade. Water was purified using Milli-Q. Standard DexP (99.5%) was purchased from Sigma Chemical Company (St. Louis, MO, USA). All solvents and sample solutions used for HPLC and HPLC-UV-ESI/MS were filtered through a 0.45 μm membrane.

Ten milligrams of DexP was dissolved in 100 ml water to yield a 100 μg/ml stock solution. The calibration curve was prepared by diluting the stock solution in the mobile phase to the final concentrations of 0.5, 1, 5, 10, 25 and 50 μg/ml for DexP.

2.2. Analysis conditions

The HPLC–MS system includes an Agilent 1100 series HPLC (Agilent Co. Ltd., Germany) and an Esquire 3000 ESI/MS with an ion trap mass spectrometer (Bruker Daltonik GmbH, Germany). The separation was carried out on a Phenomenex (Torrance, CA, USA) ODS(3) column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. The mobile phase was acetonitrile–5 mmol/l ammonium acetate (23:77 (v/v)) at a flow rate of 1.0 ml/min, and the detection wavelength was at 245 nm. The injection volume was 20 μl. The full scan electrospray ionization/mass spectra were obtained with both negative and positive polarity. The ion source temperature was 300 °C. The capillary voltage was ±4.0 kV. The nebulizer pressure was 20 psi. The nitrogen flow rate was 9 l/h. Before the eluate from HPLC was introduced into mass spectrometer, the flow rate was split 25:1. The software that was used included Bruker Daltonics EsquireControl 5.xx, DataAnalysis 2.00 and Agilent ChemStation A.07.

2.3. CPF sample preparation

2.3.1. Experimental animals

Fifty-two white cavys (170–230 g) were chosen without sex limitation from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China). They were randomly divided into group A, B and C. In group A (21 individuals), the drug was administered intravenously, while in group B (21 individuals) it was administered via middle ear. Group C (10 cavys) remained without any injection of DexP was employed as the control group.

2.3.2. Drug intake, specimen collection and preparation

2.3.2.1. Intravenous injection. The animals of group A were anesthetized with 0.06 ml 10% of chloral hydrate and 0.5% of DexP in the proportion of 400 μg DexP /100 g cavy was administered via an abdomen vein. After 10, 30 min, 1, 2, 4, 6 and 8 h, respectively, the cavy were killed. Perilymph samples were collected by taking out both cochlea, opening the hear vesicle and extracting the CPF with micro-injector through the ear membrane. The samples were immediately frozen at −20 °C. Each CPF sample was from three cavy.

2.3.2.2. Injection via middle ear. The subjects of group B were anesthetized and treated same as group A but via middle ear injection. After 30 min, the cochlea was washed six times with a total amount of 1 ml water. Then killing cavy, collecting and stocking the CPF samples were performed the same as for group A.

2.3.2.3. The normal control. The cavys of group C were anesthetized with 0.06 ml 10% of chloral hydrate. *One hour later, the cavys were killed.* Perilymph samples were collected and stocked as group A. The CPF of 10 cavys were combined to produce CPF blank.

It is noteworthy that the CPF *samples prepared as above were* comparatively clear and the volume of each CPF sample was about 100 μl. Therefore, no further sample clean-up procedure was necessary.

2.4. Recovery and reproducibility

The recovery was obtained by determining the spiked solution with CPF blank and DexP standard at three concentrations of 2, 10 and 25 μg/ml.

The reproducibility was assessed using relative standard deviations (R.S.D.s) of intra- and inter-day peak area with three concentrations of 2, 20 and 50 μg/ml for DexP.

3. Results and discussion

3.1. MS and MS^2 elucidation of DexP

The full scan ESI/MS and MS^2 of DexP were obtained with positive (Fig. 1) and negative (Fig. 2) polarity. In Fig. 1A, the base peak ion at *m/z* 495 was corresponded to $[M + 2H - Na]^+$. Signals at *m/z* 539, 517 and 473 were assigned to $[M + Na]^+$, $[M + H]^+$, $[M + 3H - 2Na]^+$, respectively. In Fig. 1B, MS^2 of the precursor ion at *m/z* 517 produced the fragment ions at *m/z* 499 and 479, corresponding to $[M+H-H_2O]^+$ and $[M+H-H_2O-HF]^+$,

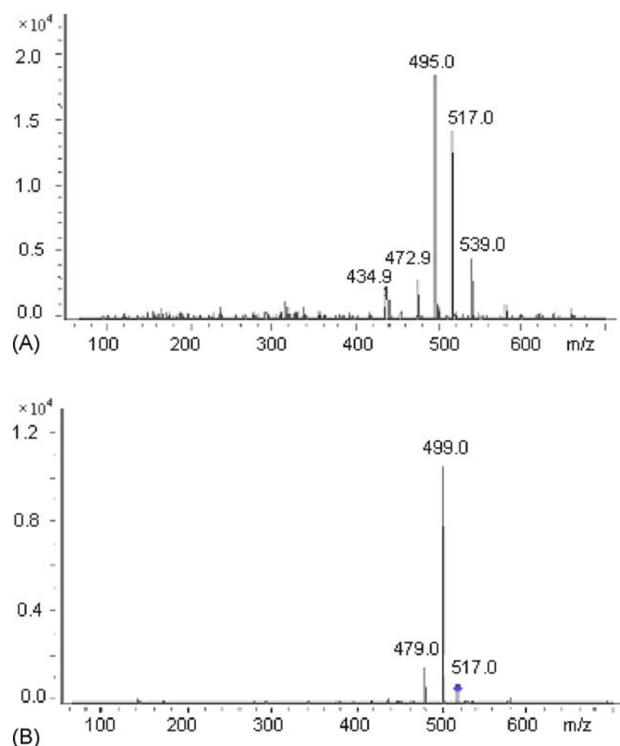

Fig. 1. ESI/MS and MS^2 of DexP under positive polarity: (A) ESI/MS and (B) MS^2.

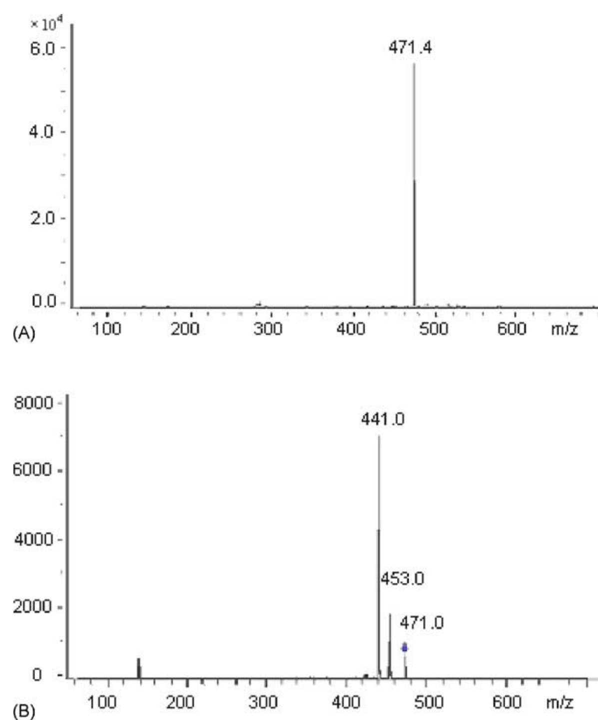

Fig. 2. ESI/MS and MS^2 of DexP under negative polarity: (A) ESI/MS and (B) MS^2.

respectively. With negative electrospray ionization, only one base peak ion at *m/z* 471 was observed in its MS (Fig. 2A), which corresponded to the quasi-molecular ion $[M+\mathrm{H}-2\mathrm{Na}]^-$. In Fig. 2B, the production ions at *m/z* 441 and 453 resulted from the precursor ion $[M+\mathrm{H}-2\mathrm{Na}]^-$, corresponding to $[M+\mathrm{H}-2\mathrm{Na}-\mathrm{CH_2O}]^-$ and $[M+\mathrm{H}-2\mathrm{Na}-\mathrm{H_2O}]^-$, respectively. The loss of formaldehyde proceeded via a possible five-membered ring transition. Under both positive and negative polarity, the possible cleavage pathways for DexP were schemed in Fig. 3. These MS and MS^2 data could provide two alternative strategies to characterize DexP. However, the negative mode produced comparatively less fragments and a higher intensity of the signals than in the positive mode. Therefore, the negative mode is obviously superior and the specific ions $[M+\mathrm{H}-2\mathrm{Na}]^-$ and $[M+\mathrm{H}-2\mathrm{Na}-\mathrm{CH_2O}]^-$ (at *m/z* 471 and 441) were employed for the subsequent characterization of DexP in the CPF sample.

3.2. HPLC separation

In the published work on analyzing DexP by HPLC, ODS(C_{18}) column and CH_3OH (CH_3CN)–water or CH_3OH (CH_3CN)–K_2HPO_4 mobile phase systems have been most widely used. The pH values of the mobile phase varied between 2 and 5 via the adjustment of H_3PO_4. Although some reports [3,11] have demonstrated good resolution and high sensitivity [3,11], some else [14,15] still suffer from the larger $W_{1/2}$ (peak width at half height), which will worsen the resolution and sensitivity. In our research, the observations (Table 1) of the retention times and $W_{1/2}$ for DexP were carried out on different columns using the same mobile phase (CH_3CN–5 mmol/l NH_4Ac (30:70 (v/v)), pH 7.4). As can be seen from Table 1, the smaller $W_{1/2}$ of DexP could only be obtained on the Phenomenex ODS(3) column. With the variation of the pH value of the mobile phase from 2 to 5 (adjusted with H_3PO_4), the similar trend can be seen to that in Table 1, i.e. the smaller $W_{1/2}$ of DexP

Table 1
Retention times and $W_{1/2}$ for DexP on the different columns[a]

Column	Part number	t_R (min)	$W_{1/2}$ (min)
Nova-PakC_{18} (150 mm × 3.9 mm)	T72391P	4.21	0.77
SupelcoLC-18 (250 mm × 4.6 mm)	018084AK	6.55	0.94
MachereyNagel C_{18} (250 mm × 4.6 mm)	1016436	7.02	0.72
Phenomenex ODS(3) (250 mm × 4.6 mm)	311308	5.92	0.32
Symmetry C_8 (150 mm × 3.9 mm)	T72102U	6.71	0.73

[a] The mobile phase was CH_3CN–5 mmol/l NH_4Ac (pH 7.4) (30:70 (v/v)) at flow rate of 1 ml/min. The UV wavelength was at 245 nm.

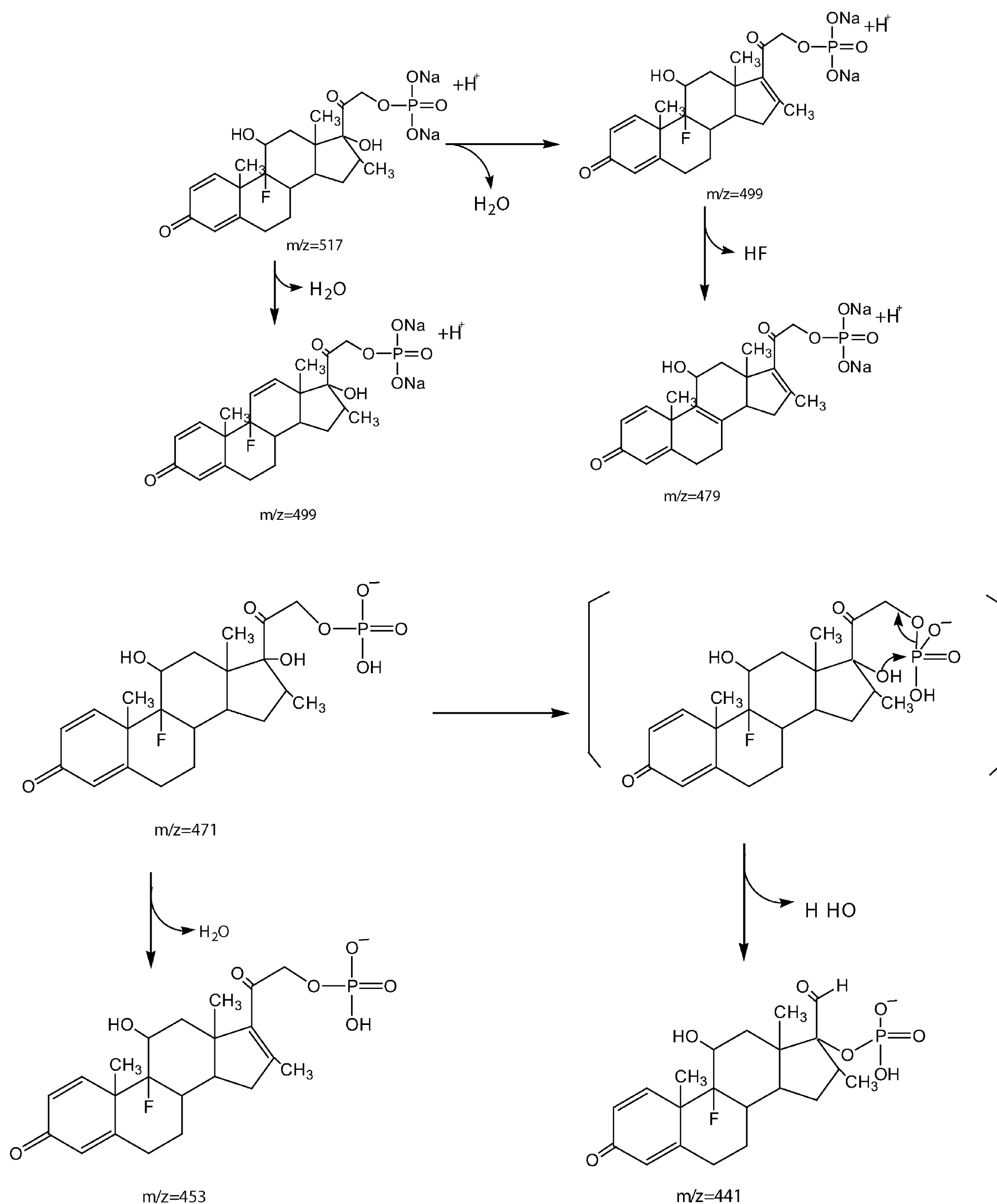

Fig. 3. Cleavage pathways of DexP.

(the data not given) was again observed on the Phenomenex ODS(3) column rather than on other else columns shown in Table 1.

Taking analysis time, $W_{1/2}$ and resolution between DexP and blank matrices into consideration, CH_3CN–5 mmol/l NH_4Ac (23:77 (v/v)) and Phenomenex ODS(3) were chosen as the optimal mobile phase and column. Under the optimal conditions, the reasonable retention time was 9 min for DexP with full baseline separation from the CPF blank matrices. Typical chromatograms were shown in Fig. 4.

3.3. HPLC-UV method evaluation

The linear response was observed over the concentration range from 0.5 to 50 μg/ml for DexP. The regression between peak area (A) and concentration (C, μg/ml) yielded the regression equation: $A = 28870C + 385$ ($n = 6$, $r = 0.9991$). The limit of detection (LOD) for DexP was found to be 0.10 μg/ml by calculating a signal-to-noise ratio of 2 (S/N = 2). For some CPF samples with concentration of DexP lower than LOD, the concentration was increased by bubbling N_2 through the solution.

The recovery was examined by determining the samples spiked with the CPF blank and DexP, and the results were listed in Table 2. The recovery ranged from 98.5 to 100.8%. The reproducibility of the method was checked with intra- and inter-day peak area changes at three different concentrations of 2, 20 and 50 μg/ml. The intra- and inter-day relative standard deviations (R.S.D.s) were between 0.7–1.3 and 1.2–3.5%, respectively.

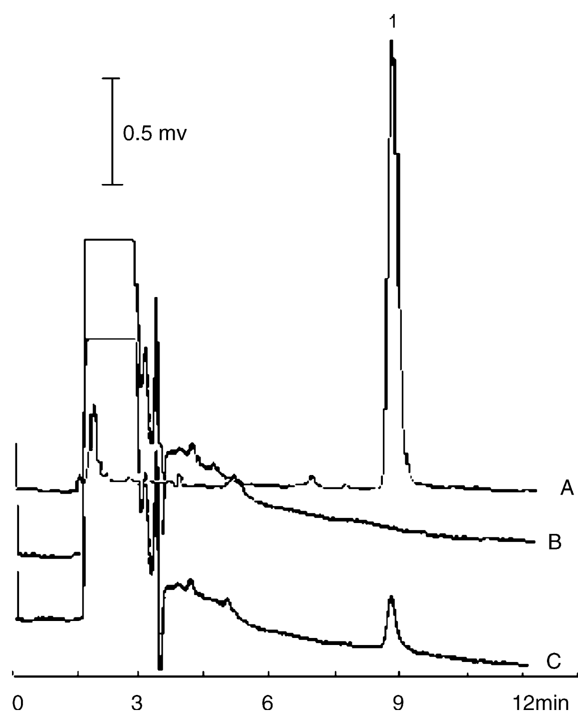

Fig. 4. Typical chromatograms conditions: column, Phenomenex ODS(3) (250 mm × 4.6 mm, 5 μm); mobile phase, acetonitrile–5 mmol/l ammonium acetate (23:77 (v/v)) (pH 7.5); flow rate, 1.0 ml/min; injection volume, 20 μl and detection wavelength, 245 nm. Chromatograms: (A) DexP standard (5 μg/ml); (B) CPF blank and (C) CPF sample. Peak (1) DexP.

3.4. Characterization of DexP by HPLC-ESI/MS

With the HPLC-ESI/MS condition described in Section 2, the total ion currents (TICs) of CPF blank, the spiked sample and CPF specimen under negative polarity were achieved. The corresponding spectra were shown in Fig. 5A–C, respectively. Fig. 5D represents the ESI/MS of substance at about 9.0 min in Fig. 5C and E is the MS^2 of the precursor ion at *m/z* 471. DexP was clearly characterized with its specific ions $[M + H - 2Na]^-$ at *m/z* 471 (Fig. 5D) and $[M + H - 2Na - CH_2O]^-$ at *m/z* 441 (Fig. 5E). As expected, at the retention time of DexP, there was no endogenous interference observed in CPF blank (Fig. 5A). Therefore, the characterization of DexP in CPF based on its specific ions ensured the results to be accurate and reliable.

3.5. Application

The developed HPLC-UV-ESI/MS method was applied to characterize and quantify DexP in CPF of cavy. Concentration–time curves were recorded (Fig. 6), and showed that the concentration of DexP in CPF of cavy reached the maximum value after 2 h when administered intravenously. Then, it gradually reduced to zero after about 6 h. After injection of DexP to the middle ear, however, the concentration of DexP in CPF of cavy reached the maximum value within 0.5 h and went rapidly down to a lower

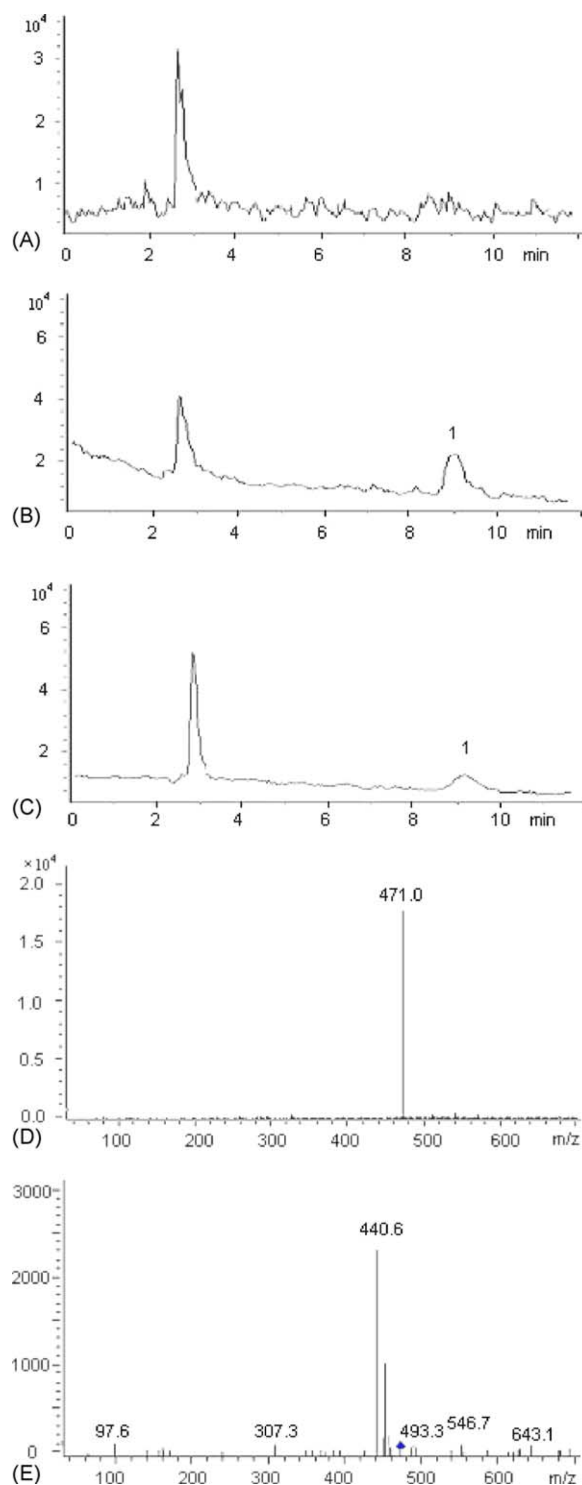

Fig. 5. Typical TIC conditions: column, Phenomenex ODS(3) (250 mm × 4.6 mm, 5 μm); mobile phase, acetonitrile–5 mmol/l ammonium acetate (23:77 (v/v)) (pH 7.5); flow rate, 1.0 ml/min; injection volume, 20 μl; splitting ratio, 25:1 and ESI polarity, negative. Spectra: (A) TIC of CPF blank; (B) TIC of the spiked sample with blank and DexP (5 μg/ml); (C) TIC of CPF specimen; (D) ESI/MS of the peak at about 9 min in CPF specimen and (E) MS^2 of the ion at *m/z* 471. Peak (1) DexP.

Table 2
The recovery Table 1

Added concentration (μg/ml)	Found concentration (μg/ml)	Average concentration (μg/ml)	Recovery (%)
2	1.90, 1.95, 2.06, 1.92, 2.02	1.97	98.5
10	10.10, 10.20, 9.85, 10.38, 9.73	10.05	100.5
25	25.87, 24.54, 25.91, 24.78, 24.90	25.20	100.8

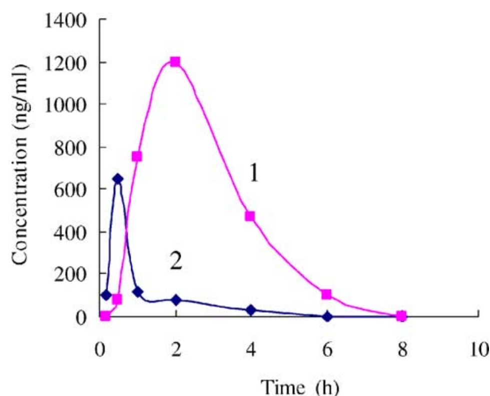

Fig. 6. Concentration–time curve of DexP in CPF of cavy curves: (1) intravenous injection and (2) injection via middle ear.

level within 1 h. Subsequently, it decreased towards zero after 6 h. Following these results, especially for the patients forbidding adrenal cortex, it is important to administer the DexP via middle ear injection. Moreover, it is noteworthy that the increase of the administered frequency will be also necessary to achieve a better curative effect.

4. Conclusion

We have described an HPLC-UV-ESI/MS method to identify and quantify DexP in CPF of cavy. Under the proposed conditions, DexP was well resolved from the matrices in CPF blank. This method possessed advantages of a good peak shape, high reproducibility and accuracy, and better sensitivity and shorter analysis time. This method has been proven suitable for routine determination of DexP in CPF of cavy. Moreover, the investigation on concentration–time curve of DexP in CPF of cavy would provide the reference for clinical pharmacokinetics study and practice.

Acknowledgements

The authors would like to thank the support of Henan Outstanding Youth Grant (04120001200) and Henan Outstanding People Innovation Grant (0421002300), and also thank Dr. Kerrie Bidwell for improving the writing of this manuscript.

References

[1] K.Z. Tang, X.J. Fu, G. Li, Chin. Hosp. Pharm. J. 22 (1) (2002) 26.
[2] A.H. Sun, L.S. Pames, D.J. Freeman, S.Z. Lin, W.P. Liu, Chin. J. Otorhinolaryngol. 36 (1) (2001) 62.
[3] D.K. Huang, P. Lei, Z.Y. Dai, Chin. Pharm. 13 (2002) 497.
[4] Q.F. Liao, Z.Y. Xie, B.L. Xie, C. Ou-Yang, Chin. J. Hosp. Pharm. 20 (2000) 539.
[5] A.S. Amin, Anal. Lett. 29 (1996) 1527.
[6] L. Ayllon, M. Silva, D. Perez-Bendito, J. Pharm. Sci. 83 (1994) 1135.
[7] X.L. Wang, Y.L. Li, Chin. J. Pharm. Pract. 1 (2) (1998) 106.
[8] V. Baeyens, E. Varesio, J.L. Veuthey, R. Gurny, J. Chromatogr. B 692 (1997) 222.
[9] M. Zignani, S. Einmahl, V. Baeyens, Eur. J. Pharm. Biopharm. 50 (2000) 251.
[10] J. Hoebus, E. Daneels, E. Roets, J. Hoogmartens, J. Planar Chromatogr. 6 (4) (1993) 269.
[11] J. Dong, G.L. Duan, Z. Liu, Chin. J. Clin. Pharm. 12 (2003) 27.
[12] Z. Milojevic, D. Agbaba, S. Eric, et al., J. Chromatogr. A 949 (2002) 79.
[13] L. Wu, Chin. Pharm. J. 31 (1996) 415.
[14] J.P. Yuan, W.D. Li, Modern Pharm. 12 (3) (1995) 35.
[15] J. Tian, X.S. Chen, R.D. Wang, Y.Q. Nie, Pharm. J. Chin. PLA 17 (2001) 334.