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Separation and characterization of the metabolic products of lappaconitine in rat urine by high-performance liquid chromatography

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SUMMARY

The separation and characterization of the metabolic products of lappaconitine in rat urine by high-performance liquid chromatography with electrochemical and ultraviolet detection are described. Urine samples from rats intravenously administered lappaconitine hydrobromide were extracted with chloroform and then purified on a Sep-Pak C_{18} cartridge. The subsequent resolution into individual compounds was achieved by high-performance liquid chromatography. Identification of these compounds was based on comparisons of the chromatographic behaviour and the detector response with those of authentic samples. Changes in the ratio of lappaconitine to its metabolites in rat urine with time after dosing led to a proposal for one of the probable metabolic pathways of lappaconitine in the rat.

INTRODUCTION

Lappaconitine (LAP) is an active alkaloid isolated from the plant *Aconitum sinomontanum* Nakai [1]. Lappaconitine hydrobromide has recently been used as an analgesic drug in China. The chemistry, toxicology and pharmacology of LAP have been reported [2–4], but its metabolism is poorly understood. Therefore, we undertook the present study of the metabolism of LAP.

Traditional techniques, such as gas chromatography–mass spectrometry (GC–MS), may be employed in the separation and characterization of drug metabolites and successfully provide the structural assignment of unknown substances. However, this method with prior purification and derivatization

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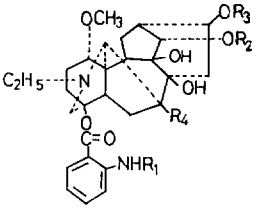

Compound	R_1	R_2	R_3	R_4
LAP	$COCH_3$	CH_3	CH_3	H
14-DMDAL	H	H	CH_3	H
16-DMDAL	H	CH_3	H	H
14,16-DDMDAL	H	H	H	H
DAL	H	CH_3	CH_3	H
I S	$COCH_3$	CH_3	CH_3	OH

Fig. 1. Structures of lappaconitine (LAP), N-deacetyl-14-O-demethyllappaconitine (14-DMDAL), N-deacetyl-16-O-demethyllappaconitine (16-DMDAL), N-deacetyl-14,16-O-didemethyllappaconitine (14,16-DDMDAL), N-deacetyllappaconitine (DAL) and ranaconitine (I.S.).

procedures is tedious and time-consuming. In addition, GC–MS is not suitable for thermally substances such as those studied here. The resolving power of high-performance liquid chromatography (HPLC) combined with the sensitivity and selectivity of electrochemical detection is able to provide the selective identification of electroactive species in complex biological samples [5–7]. Further identification may be achieved by simultaneous detection with ultraviolet (UV) and electrochemical detectors in series, which is similar to dual-electrode amperometric or UV absorbance combined with fluorescence detection [6–8]. In this paper, we describe the application of these approaches to the separation and characterization of LAP metabolites in urine from rats receiving lappaconitine hydrobromide. The structure of the compounds concerned are known in Fig. 1.

EXPERIMENTAL

Instruments

The chromatograph used for HPLC was a Model 501 (Waters Assoc., Milford, MA, U.S.A.), equipped with a Model LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and/or a Jasco Model UVIDEC-100-II UV detector (Tokyo, Japan) monitoring the absorbance at 252 nm. The applied potential of the electrochemical detector was established against an Ag/AgCl reference electrode. The test sample was introduced using a Model 7125 syringe-loading sample injector with a 100-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). HPLC was carried out on a μBondapak C_{18}

column (10 μm particle size, 30 cm×0.39 cm I.D. or 30 cm×0.78 cm I.D.) (Waters Assoc.) at 18–25°C. The pH of the mobile phase was adjusted with orthophosphoric acid and the flow-rate was set at 1.0 ml/min unless stated otherwise.

Reagents and materials

LAP, N-deacetyllappaconitine (DAL) and ranaconitine (internal standard, I.S.) were prepared from *Aconitum sinomontanum* Nakai roots in our laboratories by the methods reported previously [1,9]. For this purpose, the powdered roots of *Aconitum sinomontanum* Nakai moistened with 10% (w/v) sodium carbonate were extracted with benzene and then the benzene layer was re-extracted with 2% (w/v) hydrochloric acid. The acid solution, adjusted to pH 9.0, was back-extracted with diethyl ether. After evaporation of the solvent, the residue was recrystallized from diethyl ether to give LAP as colourless prisms, m.p. 223–224°C (literature, 224°C [1]). The mother liquor of LAP was subjected to column chromatography on aluminium oxide. Recrystallization of the residue corresponding to ranaconitine obtained from diethyl ether gave ranaconitine as colourless needles, m.p. 137–138°C (literature, 137°C [1]). Their identities were confirmed by mass spectrometry (m/z 584 for LAP and 600 for ranaconitine). DAL was extracted from the powdered roots of *Aconitum finetianum* Hand-Mazz by the same procedure and the residue corresponding to the desired compound extracted with organic solvent was subjected to column chromatography on aluminium oxide. Recrystallization of the product obtained above from light petroleum–ethyl acetate gave DAL as colourless needles, m.p. 118–119°C (literature, 117–119°C [9]). Its identity was confirmed by mass spectrometry (m/z 542). N-Deacetyl-14-O-demethyllappaconitine (14-DMDAL), N-deacetyl-16-O-demethyllappaconitine (16-DMDAL) and N-deacetyl-14,16-O-didemethyllappaconitine (14,16-DDMDAL) were kindly provided by Showa Yakuhin Kaco (Tokyo, Japan). The structures of these compounds are given in Fig. 1. A Sep-pak C_{18} cartridge (Waters Assoc.) was washed successively with methanol (10 ml) and deionized water (10 ml) prior to use. Other reagents used were of analytical-reagent grade, and solvents were purified by distillation prior to use.

Urine sample from rats

Male Wistar rats weighing approximately 200 g were intravenously administered lappaconitine hydrobromide (4 mg/kg). Three rats were housed in one cage for collection of urine, and the urine was collected at different time intervals following administration (0–6, 6–12, 12–24 and 24–48 h) and stored at −20°C until analysis.

Extraction of lappaconitine and its main metabolites from rat urine with chloroform followed by a Sep-Pak C_{18} cartridge

Amounts of 1 μg of LAP, 14-DMDAL, 16-DMDAL, 14,16-DDMDAL and DAL were added to 2 ml of control rat urine and the pH of the resulting solution was adjusted to 9.0 with 10% (w/v) ammonia solution. The solution was extracted three times with 1-ml portions of chloroform–methanol (10:1, v/v). The combined organic solvent was washed with water (2 ml) and then dried over anhydrous sodium sulphate. Evaporation of the solvent under reduced presssure gave a residue which was dissolved in 0.5% (w/v) ammonium acetate buffer (pH 3.0, 2 ml) and passed through a Sep-Pak C_{18} cartridge. After successive washing with water (2 ml) and 10% (w/v) methanol (2 ml), the desired fraction was eluted with methanol (5 ml). The effluent, after addition of I.S. (2 μg) was subjected to HPLC with electrochemical detection on a μBondapak C_{18} column (30 cm × 0.39 cm I.D.) using 0.5% (w/v) ammonium acetate (pH 5.2)–tetrahydrofuran (THF)–methanol–acetonitrile (17:2:3:1, v/v) as mobile phase and the applied potential was set at +1.0 V.

Separation and characterization of lappaconitine and its metabolites in rat urine

Urine samples (20 ml) were adjusted to pH 9.0 and extracted three times with 10-ml portions of chloroform–methanol (10:1, v/v). The residue obtained above was dissolved in 10 ml of 0.5% (w/v) ammonium acetate buffer (pH 3.0) and passed through a Sep-pak C_{18} cartridge. After successive washing with water (10 ml) and 10% (w/v) methanol (5 ml), the desired fraction was eluted with methanol (20 ml). The eluate was evaporated under reduced pressure below 40°C. Further purification was performed by HPLC on a semi-preparative column, using 0.5% (w/v) ammonium acetate (pH 5.2)–THF–methanol–acetonitrile (17:3:2:1, v/v) as the mobile phase at a flow-rate of 1.5 ml/min. The fractions with capacity factors (k') of 1.68–1.88 (I), 1.89–2.05 (II), 2.06–2.27 (III) and 2.48–2.72 (IV) corresponding to LAP, 14-DMDAL, 16-DMDAL and DAL on the chromatogram, respectively, were collected and subjected to rechromatography on an analytical column, using 0.3% (w/v) ammonium acetate (pH 5.2)–methanol–acetonitrile (11:2:4, v/v) and 0.3% (w/v) ammonium acetate (pH 5.2)–THF–methanol (10:1:2, v/v) as mobile phases, respectively. Each of compounds I–IV (ca. 20 μg of I, 10 μg of II, 15 μg of III and 20 μg of IV) was obtained as a colourless oily residue with a single peak on the chromatogram by HPLC with electrochemical detection (applied potential +1.0 V). The eluate corresponding to each peak on the chromatogram, after addition of I.S. (20 μg), was subjected to HPLC using 0.3% (w/v) ammonium acetate (pH 5.2)–THF–acetonitrile (64:9:7, v/v), 0.3% (w/v) ammonium acetate (pH 5.2)–THF–methanol–acetonitrile (26:3:2:1, v/v), 0.3% (w/v) ammonium acetate (pH 5.2)–THF–methanol (8:1:1, v/v), 0.3% (w/v) ammonium acetate (pH 5.2)–acetonitrile (5:2) and

0.3 % (w/v) ammonium acetate (pH 5.2)–methanol–acetonitrile (3:1:1, v/v) as mobile phases of different composition and 0.3% (w/v) ammonium acetate (pH 3.0, 4.0 or 5.0)–THF–methanol–acetonitrile (20:2:3:1, v/v) as mobile phases of different pH, and simultaneously monitored with UV and electrochemical detectors in series.

Examination of changes in ratios among lappaconitine and its metabolites in rat urine at different time intervals after dosing lappaconitine

Urine samples (each 2 ml) obtained at different time intervals were extracted with chloroform–methanol (10:1, v/v) and then a Sep-Pak C_{18} cartridge in the same manner as described above. Each of the samples thus obtained was subjected to HPLC with electrochemical detection on an analytical column using 0.5% (w/v) ammonium acetate (pH 5.2)–THF–methanol–acetonitrile (17:2:3:1, v/v) as mobile phase and the applied potential was set at +1.0 V.

Alkaline hydrolysis of lappaconitine and its metabolites

A 0.5% (w/v) methanolic potassium hydroxide solution of each fraction obtained above was refluxed for 1 h. After addition of 2 ml of water, the methanol was evaporated under reduced pressure. The aqueous layer was neutralized to pH 7.0 and then passed through a Sep-Pak C_{18} cartridge. After washing the cartridge with water, the hydrolysis products were eluted with methanol. The eluates were subjected to HPLC with electrochemical detection (applied potential +0.8 V), using 0.3% (w/v) ammonium acetate (pH 3.0)–THF–methanol–acetonitrile (20:2:3:1, v/v) as mobile phase. The retention time for *o*-acetaminobenzoic acid was 5.5 min and that for *o*-aminobenzoic acid 3.9 min.

RESULTS AND DISCUSSION

As the dose of LAP administered to rats was only 4 mg/kg, LAP and its metabolites in rat urine were present in very small amounts. Therefore, it was necessary to develop a clean-up procedure for their efficient extraction. In a preliminary experiment, we found that LAP, 14-DMDAL, 16-DMDAL, 14,16-DDMDAL or DAL (1 μg) was each more than 70% recovered by means of liquid-phase extraction with chloroform–methanol and then solid-phase extraction with a Sep-Pak C_{18} cartridge. On the basis of these data, the separation and characterization of LAP and its metabolites in rat urine were carried out. The eluate of urine samples from the Sep-Pak C_{18} cartridge was further purified by HPLC on a reversed-phase semi-preparative column (Fig. 2). The fractions (I–IV) corresponding to LAP, 14-DMDAL, 16-DMDAL and DAL in the chromatogram were collected and subjected to re-chromatography on an analytical column using two solvent systems.

Identification of these compounds from rat urine was then carried out. We

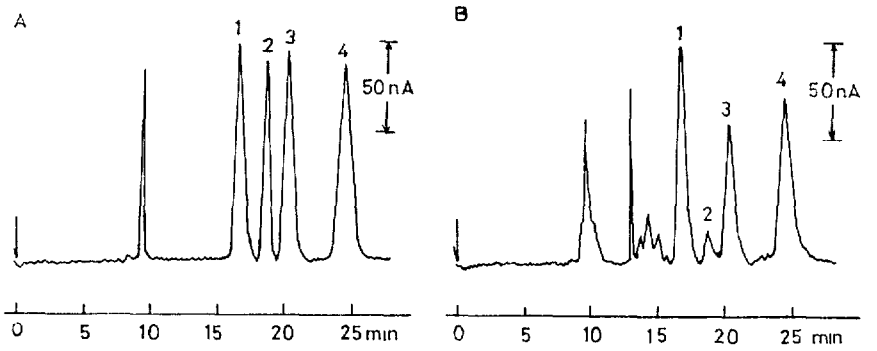

Fig. 2. Separation of lappaconitine and its metabolic products by HPLC with electrochemical detection. (A) Authentic samples; (B) sample from rat urine. Conditions: column, μBondapak C_{18} (30 cm×0.78 cm I.D.); mobile phase, 0.5% (w/v) ammonium acetate (pH 5.2)–tetrahydrofuran–methanol–acetonitrile (17 3 2·1, v/v); flow-rate, 1.5 ml/min; applied potential, +1.0 V. Peaks: 1 = LAP; 2 = 14-DMDAL; 3 = 16-DMDAL, 4 = DAL.

investigated the chromatographic behaviours with mobile phases of different composition and found that they were characteristic. The eluate corresponding to each peak on the chromatogram, after addition of the I.S., was subjected to HPLC under five conditions. The capacity factor (k') of each compound from rat urine was identical with that of the related authentic sample. As the chromatographic behaviours of these alkaloidal compounds were dependent on the pH of the mobile phase, these properties were applied to their structural characterization. It was found that the capacity factors of these compounds from rat urine were also identical with those of the related authentic samples when three kinds of mobile phase of different pH were used.

Previous studies demonstrated that the effect of electrode potential on electrochemical detector response is characteristic and therefore an aid in the characterization of the electroactive species [5–7]. In this study, a similar approach was employed. The hydrodynamic voltammograms of authentic samples and samples from rat urine were investigated. Each curve of the electrode current versus electrode potential was generated by making repeated injections of the same sample while varying the applied potential from +0.4 to +1.0 V versus Ag/AgCl. Authentic samples of 14-DMDAL, 16-DMDAL and DAL showed much higher electroactivity than LAP owing to the free aromatic amine group in their structures. These characteristic patterns were helpful for differentiating the structural features of these compounds. The hydrodynamic voltammograms of compounds I–IV were virtually identical with those of the corresponding authentic samples. These results supported the structural assignment of these compounds from rat urine.

As the UV and electrochemical responses of the compounds possessing both UV absorbance and electroactivity were different when the detection was car-

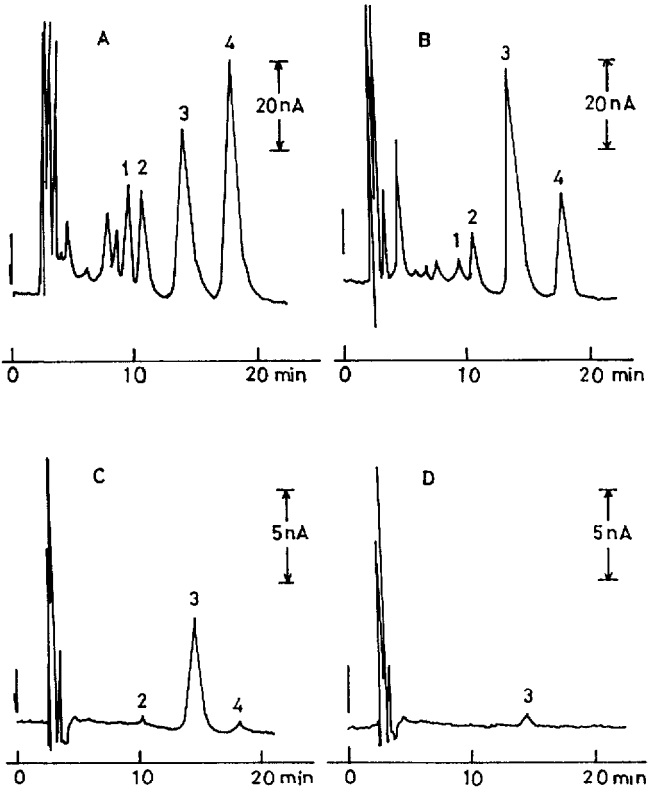

Fig. 3. Chromatograms of lappaconitine and its metabolic products in the urine from rats receiving LAP intravenously at different time intervals. Samples: (A) 0–6 h urine; (B) 6–12 h urine; (C) 12–24 h urine; (D) 24–48 h urine. Conditions: column, μBondapak C_{18} (30 cm×0.39 cm I.D.); mobile phase, 0.5% (w/v) ammonium acetate (pH 5.2)–tetrahydrofuran–methanol–acetonitrile (17·2:3:1, v/v); flow-rate, 1.0 ml/min; electrochemical detection; applied potential, +1.0 V vs. Ag/AgCl. Peaks: 1 = LAP; 2 = 14-DMDAL; 3 = 16-DMDAL; 4 = DAL.

ried out in solvent systems of different pH, further identification of compounds I–IV could be obtained. The HPLC column was connected to UV and electrochemical detectors in series and the effluents from the column were monitored by UV and electrochemical detection simultaneously. The electrochemical-to-UV detector response ratios of compounds I–IV from rat urine were identical with those of the corresponding authentic samples. The ratio of the amounts of LAP, 14-DMDAL, 16-DMDAL and DAL in 24-h rat urine after receiving LAP intravenously was calculated to be ca. 4:2:3:4. Alkaline hydrolysis of compounds I–IV resulted in the production of *o*-acetaminobenzoic acid or *o*-aminobenzoic acid, which was unambiguously characterized by HPLC with electrochemical detection.

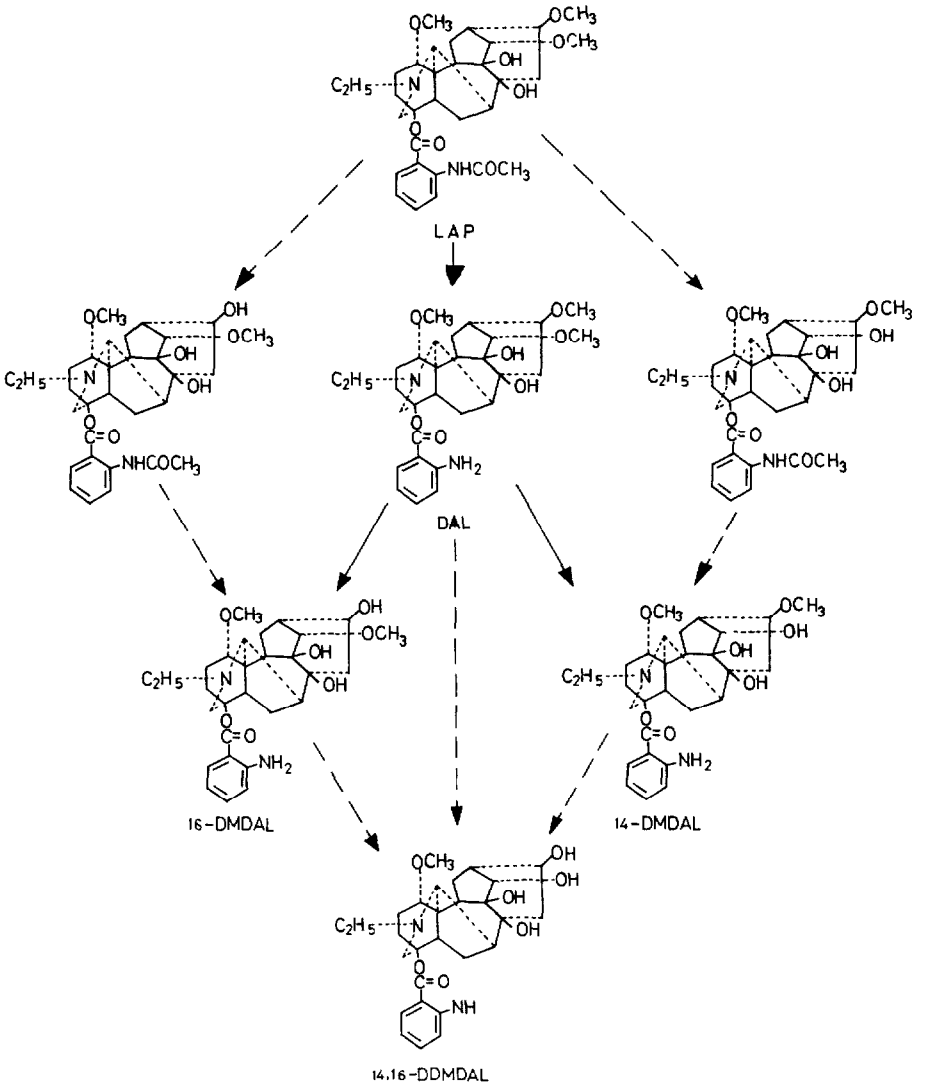

Fig. 4. Possible metabolic pathway of lappaconitine in the rat. Dashed line represents the possible metabolic pathways without any evidence from this study

It is evident from these results that LAP, 14-DMDAL, 16-DMDAL and DAL are present in the urine from rats receiving LAP.

The amounts of compounds I–IV in rat urine at different time intervals over a period of 48 h following administration of LAP where then examined. As shown in Fig. 3, the urine level of LAP decreased gradually, whereas that of DAL increased gradually relative to that of LAP. This suggested that LAP was metabolized to DAL by N-deacetylation. However, when the urine level of DAL

decreased, those of 14-DMDAL and 16-DMDAL increased, that is, the urinary ratios of 14-DMDAL and 16-DMDAL relative to DAL increased gradually with time. It seems most likely that 14-DMDAL and 16-DMDAL were formed from DAL by O-demethylation. Of course, it may be considered that there are other pathways for the formation of 14-DMDAL and 16-DMDAL, e.g., LAP was first O-demethylated and then N-deacetylated, or LAP underwent O-demethylation and N-deacetylation simultaneously. The results of this study, however, do not support such an interpretation. Because a peak corresponding to 14,16-DDMDAL could not be obtained, in spite of our efforts, the formation of this compound was not established. Taking these results into consideration, one of the probable metabolic pathways of LAP in rat is that proposed in Fig. 4. The parent drug LAP and its metabolites were hardly detectable after 24 h following administration of LAP, suggesting that LAP was metabolized and excreted rapidly in the rat.

Comparison of the amounts of LAP and its metabolites extracted from rat urine before and after hydrolysis with β-glucuronidase and sulphatase at 37°C for 12 h showed no significant differences, indicating that conjugation with glucuronic acid and sulphuric acid in rat was not an important metabolic pathway.

The biological activity of DAL, one of the metabolites of LAP, was studied after administration of this compound isolated from *Aconitum* roots in rats and mice [10]. It was found that this metabolite has marked analgesic, anti-inflammatory and antipyretic activities, similarly to the parent LAP. However, DAL exhibits marked local anaesthetic activity, which is different from LAP. In addition, DAL possesses a broader therapeutic range than LAP in experimental rats and mice.

This is the first report on the metabolism of LAP in the rat. Further studies on the metabolism of LAP in humans are being conducted, and some of the results will be reported elsewhere [11].

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REFERENCES

1 B.Y. Wi, S.W. Kong, Z.Y. Zhou, H.C. Wang and R.H. Zhu, Zhongyao Tongbao, 6 (1981) 26.
2 F. Dybing, O. Dybing and K.B. Jensen, Acta Pharmacol. Toxicol., 7 (1951) 337.
3 X.C. Tang, M.Y. Zhu, J. Feng and Y.E. Wang, Acta Pharm. Sin., 18 (1983) 579.
4 M. Ono and T. Satoh, Arzneim.-Forsch., 38 (1988) 892.
5 K. Shimada, F. Xie, T. Niwa, T Wakasawa and T. Nambara, J. Chromatogr., 400 (1987) 215.

6 M. Hamilton and P.T. Kissinger, Drug Metab. Dispos., 14 (1986) 5.
7 A.E. Rottero and P.T. Kissinger, Biomed. Chromatogr., 2 (1987) 24.
8 S.P. Assenza and P.R. Brown, J. Chromatogr., 289 (1984) 355.
9 S.H. Jiang, Y.L. Zhu, Z.Y. Zhou and R.H. Zhu, Acta Pharm. Sin., 18 (1983) 440.
10 J.H. Liu, Y.X. Zhu and X.C. Tang, Acta Pharmacol.. Sin., 8 (1987) 301.
11 F. Xie, H.C. Wang, J.H. Li, H.L. Shu, J.R. Jiang, J.P. Chang and Y.Y. Hsieh, Biomed. Chromatogr., 4 (1990) in press.