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Simultaneous quantitative analysis of methyl salicylate, ethyl salicylate and salicylic acid from biological fluids using gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric (GC–MS) assay was developed for the quantitative analysis of methyl salicylate (MeS), ethyl salicylate (ES) and salicylic acid (SA) from biological fluids. The method was validated from 100-μl rat liver homogenate preparations (5 mg/ml protein) in 70 m*M* KH_2PO_4 (pH 7.4) buffer and from 100 μl rat plasma. The samples were extracted with chloroform, derivatized with BSTFA and quantitated by GC–MS in the SIM mode. The standard curves ranged from 31 ng/ml to 800 or 1250 ng/ml. Relative standard deviations and bias were less than 11% in plasma and homogenate for all compounds except SA which evidenced greater variability. The assay was used in preliminary experiments to characterize the pharmacokinetics of MeS in rats.

Keywords: Methyl salicylate; Ethyl salicylate; Salicylic acid

1. Introduction

Methyl salicylate (MeS) (oil of wintergreen) is used for relieving muscle aches and pains by applying externally to the affected area. It is an ingredient in numerous commercially available products, such as Ben-Gay, and is available without a physician's prescription as a 100% pure liquid. Due to its sweet smelling aroma (candy-like) it is attractive to small children and is a frequent cause of poisoning [1–4]. MeS is converted to salicylic acid (SA) in the body. Virtually all studies of MeS toxicity, absorption or disposition quantitate SA in human or animal plasma. There have been no studies, to the best of our knowledge, which have attempted to characterize MeS pharmacokinetics following oral MeS dosing.

The objective of this study was to develop a method of analysis for detecting and quantifying MeS, ethyl salicylate (ES) and SA from biological fluids of the rat. The assay also permits evaluation of the potential hepatic transesterification of MeS in the presence of ethanol.

2. Experimental

2.1. Chemicals and reagents

All chemicals and solvents used were of analytical or gas chromatography (GC) grade. MeS, propyl

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paraben (PP) and ethyl paraben (EP) were purchased from Sigma (St. Louis, MO, USA). SA and ES were obtained from J.T. Baker (Phillipsburg, NJ, USA) and ICN Biomedical (Aurora, OH, USA), respectively. Chloroform and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from EM Science (Gibbstown, NJ, USA) and Pierce (Rockford, IL, USA), respectively. Deionized water used in making aqueous solutions, was produced using Millipore Milli-Q purification system (resistivity, 18.2 MΩ cm). All standards and stock solutions were made fresh daily.

2.2. GC–MS analysis

A Hewlett-Packard GC (5890 Series II) with an attached autosampler (7673A) and mass detector (5972) were used throughout the study. A 2-μl volume of solution was injected in the splitless mode onto either a 25 m×0.20 mm I.D., 0.33 μm film thickness HP-5 or a 30 m×0.32 mm I.D., 0.25 μm film thickness Simplicity-5 (Supelco) capillary column with 5% phenyl methyl silicone stationary phase. Helium was used as the carrier gas with flow-rates of 0.56 and 1.7 ml/min for the HP-5 and Simplicity-5 columns, respectively. Injector and detector temperatures were set at 250°C and 280°C, respectively. Oven temperature was initially set at 100°C, held at that temperature for 1 min and increased at a rate of 15°C/min until 280°C, where it was held for 2 min. Total run time was 15 min. The mass spectrometer was run in single ion monitoring (SIM) mode for two ions for each compound except for SA where three ions were monitored. The following ions were monitored for the five compounds: MeS, 179, 209; ES, 195, 223; SA, 209, 267 (249 was added for plasma assay); PP, 193, 210; EP, 223, 238.

For the HP-5 column the retention times were 7.3, 7.9, 8.2 and 9.5 min for MeS, ES, SA and PP, respectively. For the Simplicity-5 column the retention times were 5.5, 6.1, 6.4, 6.9 and 7.6 min for MeS, ES, SA, EP and PP, respectively. Ratios of peak areas of compounds to internal standard (I.S.) were plotted against the concentrations of the compounds to generate standard curves. Standard curve calibrations and sample quantification were obtained with use of either Excel or Chemstation.

2.3. Sample processing

A sample (100 μl) of liver homogenate (5 mg/ml protein) was added to an amber crimped glass tube containing dried NaF (20 μl saturated aqueous solution). Twenty-five μl deionized water and 25 μl (2 μg/ml) internal standard (PP) in deionized water were added to the sample and vortexed. Twenty-five μl of 3 *M* HCl was added to the mixture and vortexed. The acid was added to improve the extraction of SA. Fifty μl chloroform was added to the mixture which was then capped and crimped and shaken on a horizontal shaker for 10 min. The resulting mixture was centrifuged at 7200 *g* for 1.5 min. Twenty μl chloroform extract was transferred to another crimped amber glass tube and 20 μl BSTFA was added. The tube was capped and vortexed. The resulting solution was left overnight (approximately 16 h) at room-temperature away from light in order for derivatization to proceed. The resulting samples were transferred into screw top amber vials which were loaded into the autosampler attached to the GC–MS system.

Plasma samples (100 μl) were treated in the same manner described above for homogenate samples except that HCl was not added. Acid was deleted in this first extraction in order not to extract SA. The chloroform layer (not evaporated) was assayed for MeS and ES. The aqueous layer which remained after the initial chloroform extraction was diluted 250-fold with water. The aqueous layer (100 μl) was transferred to another crimped amber glass tube and assayed for SA. The solution was treated the same way as the homogenate samples, as outlined above, except that the I.S. added was 25 μl EP (1 μg/ml). Thirty μl of the chloroform layer was transferred to an amber crimped vial and evaporated. After evaporation of chloroform, 30 μl BSTFA was added, the cap was crimped and the mixture vortexed. The sample was assayed for SA approximately 16 h later.

Prior to validation, various conditions were evaluated in order to optimize recovery and quantitation. Extraction efficiency was examined as a function of time of shaking. The influence of time and temperature on the completeness of derivatization were studied as was the effect of NaF on MeS stability.

The assay was validated daily for three days. On each day a standard curve and a set of three

standards were assayed in triplicate. The concentrations used for the three sets were: 62.5, 250 and 625 ng/ml, representing low, medium and high sample values, respectively.

2.4. Preliminary animal study

Male Sprague–Dawley rats weighing 300–350 g were used. The right external jugular vein was cannulated two days before the study. Two hundred mg MeS was added to the dosing syringes, which contained 1 ml water and administered to the rat by oral gavage. After dosing the rats, in the two oral studies, the gavage needles and the dosing syringes were washed with methanol and the washings assayed to calculate the amount of MeS left (i.e., not administered), which was then subtracted from 200 mg to calculate the exact dose administered to the animals. This procedure was necessary since MeS is very hydrophobic and we found it impossible to accurately administer to the rats the amount of MeS initially placed in the dosing syringe. The actual doses of MeS which the animals received were 130.9 and 122.6 mg. One intravenous (i.v.) dose study was conducted in which 20 mg MeS was injected into the jugular vein as a solution in 100 μl polyethylene glycol (PEG) 400. Blood samples (0.3 ml) were collected via the indwelling jugular catheter before and at selected times until 12 or 24 h after the dose. Plasma was obtained and either stored frozen (i.v. study) or immediately assayed (oral studies).

3. Results

To estimate the time needed and the optimal procedure for shaking to efficiently extract MeS, comparisons were made between vortexing (1 and 2 min) and shaking in a horizontal shaker for 5 min. Shaking for more than 5 min gave good recovery and, therefore, to maximize efficiency of extraction, samples were shaken for 10 min.

It took about 30 min to complete the entire extraction procedure. Stability of MeS (and assuming similar behavior of ES) was determined in plasma samples for 30 min with and without addition of NaF. Degradation was negligible during 30 min and NaF had no effect (ca. 2% degradation) and, therefore, NaF was not added to the plasma samples. There was similar recovery of MeS from water and plasma but this was not true of the I.S.. As a result, the MeS/I.S. ratio was larger from plasma than water. The extraction was consistent, however, from different spiked plasma samples.

To estimate the time and temperature necessary for derivatization, chloroform extracts from low and high plasma concentrations were derivatized under the following conditions: 60°C, 20 min; 75°C, 15 min; 45°C, 30 min; 45°C, 45 min. The following criteria were used in evaluating the results: highest peak area; linear standard curve with good r^2; widest range of concentrations in the standard curve. None of these combinations provided optimal temperature and time conditions for derivation of all three compounds. Derivatization, therefore was allowed to proceed at room temperature (for approximately 16 h). Under these conditions the slopes of standard curves obtained from the peak area ratios of MeS, ES and SA to I.S. were similar.

Standards ranged from 31 ng/ml to 800 ng/ml for MeS, ES and SA in liver homogenate. Sample chromatograms of liver homogenates extracted for MeS, ES and SA are shown in Fig. 1A and 1B. For plasma the range of the standard curve was 31 to 800 ng/ml for MeS and ES and 31 to 1250 ng/ml for SA. Standard curves were validated over three days. Sample chromatograms of plasma extracted for MeS and ES are shown in Fig. 1C and 1D and a chromatogram for SA is shown in Fig. 1E. Three replicates of three standards (62.5, 250, 625 ng/ml), representing low, medium and high concentrations on the standard curve, were compared over three days to determine intra-day and inter-day reproducibility. Standard curves had r^2 values greater than 0.99. The results of the validation from homogenate extracts are shown in Table 1. The relative standard deviation (R.S.D.) and bias (%) for the low, medium and high standards were within 10% for MeS and ES. SA standards were within 20% for the low concentration and within 10% for the medium and high concentrations. Table 2 summarizes the results from the validation from plasma extracts. R.S.D. and bias were within 11% for all of the standards for all three compounds.

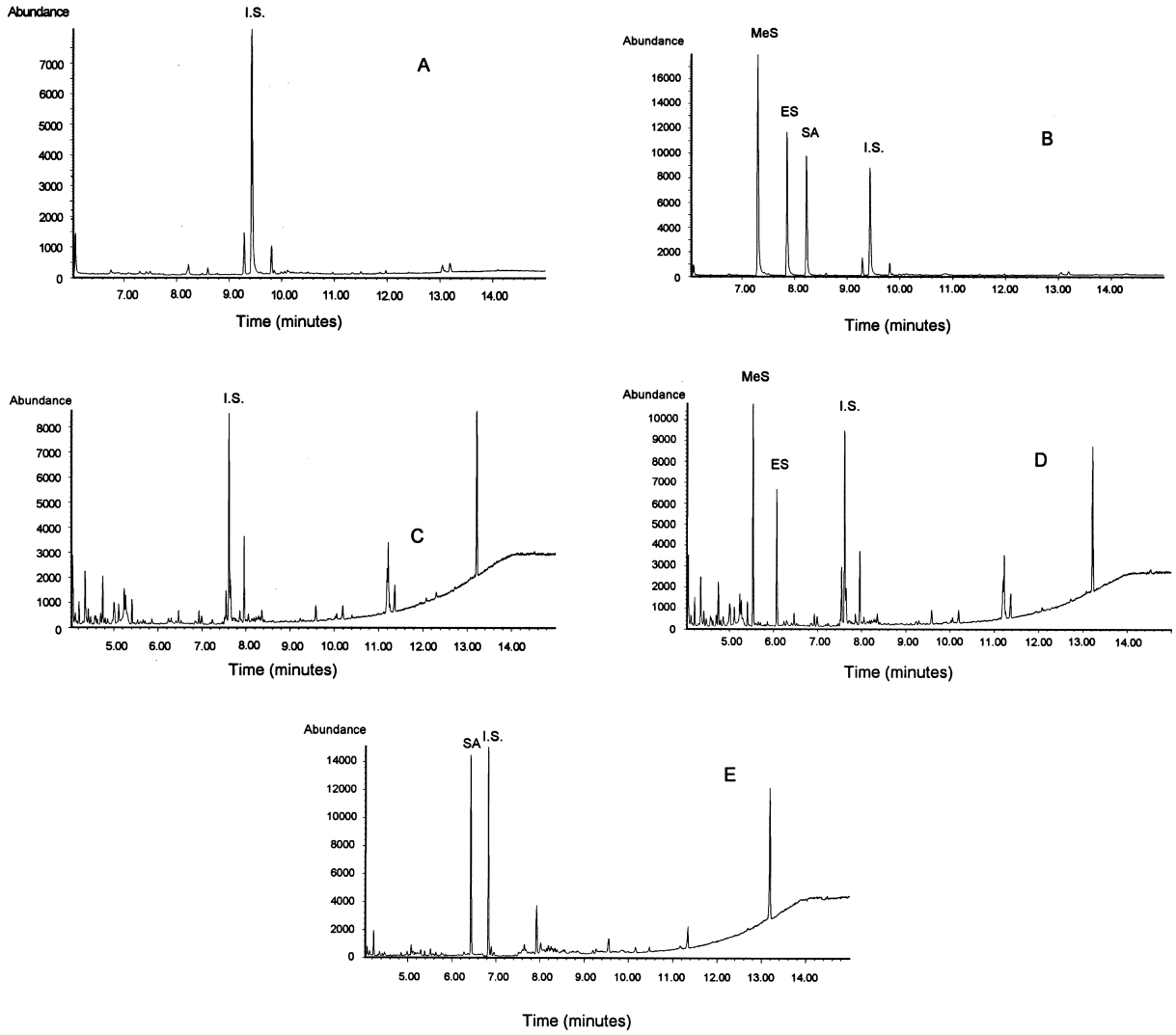

Fig. 1. (A) Mass chromatogram of a 100-μl liver homogenate extract taken through the assay. The homogenate was spiked with I.S. only. (B) Mass chromatogram of a 100-μl liver homogenate extract taken through the assay. The homogenate was spiked with 500 ng/ml of each compound. (C) Mass chromatogram of a 100-μl plasma extract taken through the assay. The plasma sample was spiked with I.S. only. (D) Mass chromatogram of a 100-μl plasma extract taken through the assay. The plasma sample was spiked with 250 ng/ml of each compound. (E) Mass chromatogram of a 100 μl plasma extract taken through the assay. The plasma sample was spiked with 250 ng/ml of each compound. Chromatograms A and B used a HP-5 column and chromatograms C–E used a Simplicity-5 column.

When dosed intravenously, the MeS plasma concentrations decreased rapidly with time when compared to the profile following oral dosing. A complete pharmacokinetic profile of MeS was obtained from the oral dosing experiment. Even though plasma was collected for 24 h, the SA concentrations did not decline sufficiently to afford a complete profile. Samples need to be collected for at least 36 h in order to properly characterize the pharmacokinetics of SA, a metabolite of MeS.

Table 1
Validation of assay in liver homogenate

Concentration added (ng/ml)	Intra-day reproducibility			Inter-day reproducibility		
	Concentration found[a]			Concentration found[b]		
	ng/ml	R.S.D. (%)	% Bias	ng/ml	R.S.D. (%)	% Bias
Methyl salicylate						
62.5	62.7	3.60	0.37	63.9	6.76	2.30
250	254	3.30	1.62	257	4.57	2.88
625	640	1.37	2.45	655	6.38	4.76
Ethyl salicylate						
62.5	59.1	0.80	−5.39	60.3	4.64	−3.49
250	263	3.85	5.37	253	5.00	1.05
625	676	2.65	8.22	680	7.99	8.88
Salicylic acid						
62.5	59.7	13.9	−4.44	58.5	19.9	−6.39
250	253	2.07	1.06	243	10.8	−2.73
625	641	5.74	2.61	639	8.69	2.24

[a] Mean of three samples. R.S.D.=Relative standard deviation (%). % Bias=[(Found−Added)×100]/Added.
[b] Mean of nine samples.

Table 2
Validation of assay in rat plasma

Concentration added (ng/ml)	Intra-day reproducibility			Inter-day reproducibility		
	Concentration found[a]			Concentration found[b]		
	ng/ml	R.S.D. (%)	% Bias	ng/ml	R.S.D. (%)	% Bias
Methyl salicylate						
62.5	63.8	2.03	2.14	67.7	4.52	8.38
250	244	0.06	−2.25	245	2.44	−2.14
625	681	5.95	8.90	674	3.50	7.76
Ethyl salicylate						
62.5	60.8	3.54	−2.65	66.8	6.64	6.85
250	232	4.74	−7.14	225	4.26	−10.0
625	665	4.28	6.47	662	3.87	5.93
Salicylic acid						
62.5	68.0	7.33	8.85	63.5	9.21	1.66
250	263	0.60	5.13	258	11.0	3.42
625	620	3.86	−0.76	619	6.56	−0.97

[a] Mean of three samples. R.S.D.=Relative standard deviation (%). % Bias=[(Found−Added)×100]/Added.
[b] Mean of nine samples.

4. Discussion

Prior to the development of quantitative chromatographic procedures, especially high-performance liquid chromatography (HPLC) and GC, SA and MeS were quantitated by colorimetry following the reaction of salicylate with a color-forming complexing agent [5,6]. These assays were not particularly sensitive nor were they specific. The color-forming complexing agent reacts with other compounds, such as phenothiazines [7]. HPLC was used for quantitating MeS and SA in three studies [7–9]. In two of those studies there was less sensitivity than was needed and both methods required conversion of MeS to salicylate for quantification [7,8]. Another study quantified MeS and ES using HPLC [10] following evaporation using a Rotavapor.

The procedure reported here, based upon recovery of MeS from a 0.1-ml biological fluid sample, has a sensitivity comparable to those reported by others [9,10]. The evaporation of chloroform in the SA assay had no effect on the analytical reproducibility. In marked contrast, however, any attempt at concentrating an organic solution of MeS (or ES) by evaporation, inevitably led to significant and erratic loss of MeS. Preventing evaporation of MeS proved to be difficult, even when samples were kept on ice. For this reason, considerable care needs to be exercised in the handling of MeS-containing solutions.

The limit of quantitation was set to be just above 30 ng/ml for all three compounds, which was well above the limit of detection. Initially, there was difficulty in obtaining reproducible and accurate values from the triplicate SA spiked plasma standards. Removing the solvent blanks (chloroform), placed after every three samples, improved the reproducibility. The solvent blanks were injected during the run to clean the injection liner, but for some reason, for which we have no explanation, this procedure created a problem with reproducibility.

MeS and ES are known to undergo chemical hydrolysis to SA. Therefore, to prevent non-enzymatic SA formation during storage, the liver homogenate and plasma samples were extracted immediately after collection.

The analytical method was applied in preliminary studies to characterize the pharmacokinetics of MeS.

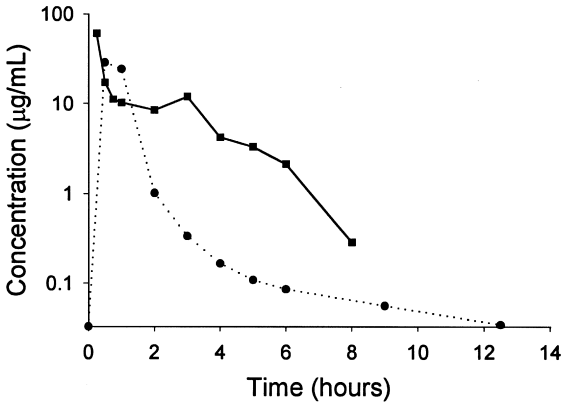

Fig. 2. Plasma concentration–time (semi-log axes) profiles of MeS in rats following an i.v. (MeS, 20 mg) (■) and an oral dose (MeS, 130.9 mg) (●).

The plasma concentration–time profile resulting from an i.v. bolus dose of MeS (Fig. 2) suggested an initial rapid distribution followed by a slower, apparent first-order terminal disposition phase. Based upon this single data set, MeS has an apparent half-life ($t_{1/2}$) of 36 min and a systemic clearance (CLs) of 14 ml/min kg.

The plasma concentration–time profile resulting from an oral dose of MeS (Fig. 2) suggested an initial increase in plasma concentrations until 15 min followed by an initial rapid decline and then a slower elimination phase. The bioavailability or fraction of the oral dose of MeS which is absorbed is estimated to be about 8%.

Preliminary studies to characterize the pharmacokinetics of SA following an oral dose of MeS (Fig. 3) suggested an initial increase in plasma concentrations until 1 h and little change in concentrations until 4 h. That pattern was followed by a gradual decline in plasma concentrations of SA which may be described as being an apparent first-order process. Based upon this single oral data set, SA has a $t_{1/2}$ of about 14 h which is similar to a $t_{1/2}$ of 10.0 h reported by Hirate et al. [11]. In the latter study an i.v. dose (173 mg/kg) of SA was administered which resulted in plasma concentrations similar to those found in this study. The investigators found that the $t_{1/2}$ increased from 3.1 to 10.0 h for SA doses of 10 and 173 mg/kg, respectively, suggesting dose-dependent kinetics. However, Hirate et al. collected plasma samples for only up to 8 h as

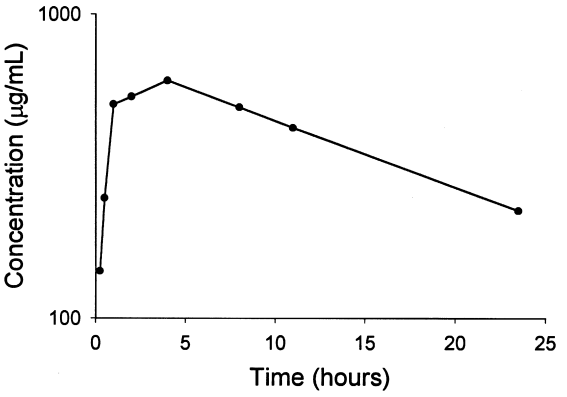

Fig. 3. Plasma concentration–time (semi-log axes) profile of SA in one rat following an oral dose of MeS (122.6 mg) (●).

compared to 24 h in this study. The plasma salicylate concentrations during 24 h evidenced relatively little change (ca. 600 to 225 μg/ml). As a consequence, although the display of the data suggests a log–linear straight line in an apparent terminal phase, this may not be the case since salicylate is known to undergo non-linear, saturable elimination. Blood samples need to be collected for a longer time after dosing (ca. 36 or 48 h) to make accurate conclusions about salicylate elimination kinetics.

While not a part of the present in vivo study, we included in this assay quantitation of ES. The reason for this is preliminary data from this laboratory indicating that MeS in the presence of ethanol undergoes transesterification to ES. This reaction is similar to the transesterification noted for cocaine [12], cocaethylene [13], meperidine and methylphenidate [14] in the presence of ethanol.

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References

[1] E.L. Liebelt, M.W. Shannon, Pediatr. Emerg. Care 9 (1993) 292.
[2] D.L. Howrie, R. Moriarity, R. Breit, Pediatrics 75 (1985) 869.
[3] J.L. Kloss, C.R. Boeckman, Ohio State Med. J. 63 (1967) 1064.
[4] M.A. Halle, P.J. Collipp, New York State J. Med. 69 (1969) 1788.
[5] P.J. Hanzlik, N.C. Wetzel, J. Pharmacol. Exp. Ther. 14 (1919) 43.
[6] C. Davison, E.F. Zimmerman, P.K. Smith, J. Pharmacol. Exp. Ther. 132 (1960) 207.
[7] B. Levine, Y.H. Caplan, J. Anal. Toxicol. 8 (1984) 239.
[8] P. Morra, W.R. Bartle, S.E. Walker, S.N. Lee, S.K. Bowles, R.A. Reeves, Ann. Pharmacother. 30 (1996) 935.
[9] A.J. Collins, L.J. Notarianni, E.F.J. Ring, M.P. Seed, Ann. Rheumatic Dis. 43 (1984) 411.
[10] H. Behrendt, H.G. Kampffmeyer, Xenobiotica 19 (1989) 131.
[11] J. Hirate, Y. Kato, I. Horikoshi, S. Nagase, C.T. Ueda, Biopharm. Drug Dispos. 10 (1989) 299.
[12] R.A. Dean, C.D. Christian, R.H.B. Sample, W.F. Bosron, FASEB J. 5 (1991) 2735.
[13] J.A. Bourland, D.K. Martin, M. Mayersohn, Drug Metab. Dispos. 26 (1998) 203.
[14] J.A. Bourland, D.K. Martin, M. Mayersohn, J. Pharm. Sci. 86 (1997) 1494.