ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1893–1901

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A direct technique for the simultaneous determination of 10 drug candidates in plasma by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry interfaced to a Prospekt solid-phase extraction system

Debra A. McLoughlin *, Timothy V. Olah, John D. Gilbert

WP26–354, Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486, USA

Received 10 October 1996; accepted 6 November 1996

Abstract

New drug candidates are being synthesized at an ever increasing rate and, until recently, the pharmacokinetics of only a few of these could be evaluated. Our laboratory is taking a novel approach to rapid multiple pharmacokinetic screening of potential drug candidates in which mixtures of new substances are co-administered to animals and analyzed simultaneously in plasma using liquid chromatography with tandem MS/MS detection in conjunction with a Prospekt automated on-line solid-phase extraction system. Plasma is sampled via an autosampler and extracted by the Prospekt with the eluent being introduced directly via a reverse phase HPLC column and a heated nebulizer interface to the mass spectrometer. Generic extraction and chromatographic conditions generally give good recoveries. The chromatographic run-times are less than 8 min. The accuracy and precision of these assays are carefully controlled with recoveries generally in the range 80–120% and coefficients of variation less than 20%. Lower quantifiable limits range from 2.5 to 5 ng ml^{-1}. This approach considerably reduces the number of animals needed to screen drug candidates and its power is illustrated by determination of the pharmacokinetics of 10 substances after their simultaneous administration to dogs. © 1997 Elsevier Science B.V.

Keywords: Plasma; Multiple analytes; Pharmacokinetic screens; LC-MS/MS; Prospekt; Automation; Solid-phase extraction; Quality control; Animal reduction

1. Introduction

The remarkable impact that HPLC with atmospheric pressure ionization tandem mass spectrometry (API-MS/MS) has had on quantitative bioanalytical chemistry within the pharmaceutical

* Corresponding author. Tel.: +1 215 6522117; fax: +1 215 6528548.

PII S0731-7085(96)02011-0

industry has recently been reviewed [1]. The unparalleled resolving power of the MS/MS detector enables the detection and independent quantitation of substances (e.g. isotopes) which cannot be chromatographically resolved. Although superior chromatographic resolution is obtained using mass-spectrometry in conjunction with capillary gas chromatography the latter technique suffers from its general incompatibility with the majority of drug candidates now being developed.

The capabilities of LC-MS/MS is now being used to conduct pharmacokinetic screening on drug candidates emanating from drug discovery groups. Typically such screening is conducted on ten new substances given simultaneously to dogs. The chromatographic run time is less than 8 min. The rate determining step in the assay procedure is the sample preparation. Many solid-phase extraction procedures have been automated using, for example, a Gilson ASPEC XL which has proved reliable and reproducible. The ASPEC XL and other robotic equipment are somewhat slow, however, and may require extracts to be evaporated and reconstituted in a mobile phase prior to analysis by LC-MS/MS [2–5]. On-line sample preparation using column switching has been reported by a number of laboratories [6]. This technique is, however, limited due to the continuous re-use of the pre-column. The Prospekt, introduced by Spark-Holland, is an automated on-line solid-phase extraction system, which eliminates this problem by processing each sample using individual disposable pre-columns [7] and eliminates the possibility of cross-contamination. By synchronizing the extraction procedure with the chromatographic run-time, a sample may be extracted while the previous one is being analyzed. Hence, samples are assayed by direct injection of plasma essentially on-line with the LC-MS/MS instrument which greatly expedites sample throughput. This paper reports experiences with this powerful combination of extraction and analytical technologies using a mixture of ten drug candidates administered simultaneously both orally and intravenously to dogs. The identity of these substances and the therapeutic class for which they were being developed cannot be disclosed but otherwise a full description of the methodology is provided.

2. Experimental

2.1. Materials

The drug candidates used in this investigation were synthesized at Merck Research Laboratories, West Point, PA. Trifluoroacetic acid (TFA) was obtained from Sigma (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Fisher (Fair Lawn, NJ).

IST Isolute solid-phase extraction CN cartridges (2.0 mm i.d. × 10 mm) were obtained from Jones Chromatography (Lakewood, CO). Liquid nitrogen and argon (99.999%) were purchased from West Point Supply (West Point, PA). Disposable vials and press-on caps were purchased from Scientific Resources (Somerset, NJ).

2.2. Dosing solutions

For oral administration, a combined dosing solution was prepared in 0.05M citric acid. The concentration of each of the ten test substances was 0.2 mg ml^{-1} of the free base. The dogs received 5 ml of dosing solution per kg of body weight. The dose of each compound was 1 mg kg^{-1}.

For intravenous administration, a combined dosing solution was prepared at a concentration of 5 mg ml^{-1} of test substance as its free base in dimethyl sulfoxide. Each dog received 0.1 ml kg^{-1} of this mixture. The dose of each compound was 0.5 mg kg^{-1}.

2.3. Animals

Four male beagle dogs (body weight ca. 10 kg) were deprived of food for 18 h prior to dosing and for 8 h postdose. Blood samples (approximately 6 ml) were collected prior to dosing and at 10, 20, 30, 40 min, 1, 1.5, 2, 3, 4, 5, 6, 8 and 24 h postdose. Plasma was harvested by centrifugation from heparinized vacutainers. Plasma samples were frozen at −20°C until taken for analysis.

2.4. Analytical method

Standard stock solutions of ten test substances and a structurally-related internal standard were prepared as 1 mg ml^{-1} solutions (of the free bases) in 50:50 acetonitrile–water containing 0.1% TFA.

Analytical standards and quality control samples were prepared by adding known volumes (0.05 ml) of combined standard solutions to 0.5 ml aliquots of control plasma. The concentrations range for each test substance was 2.5–1000 ng ml^{-1}. Six replicate quality control samples were prepared at low, middle and high concentrations. Plasma samples (0.5 ml) were pipetted into autoinjection vials. The internal standard solution was diluted to a working concentration of 500 ng ml^{-1}. 50 μl aliquots were added to each plasma sample which were then capped, vortexed and loaded onto the Triathlon autosampler (Jones Chromatography). Each sample was individually processed on the Prospekt automated on-line solid phase extraction system (Spark-Holland, Emmen, Netherlands) using the following procedure: precondition the IST Isolute CN cartridge for 30 s at 5 ml min^{-1} with acetonitrile, followed by distilled water (45 s at 6 ml min^{-1}). The plasma sample (50 ml) was loaded onto the cartridge and washed with distilled water for 80 s at a rate of 0.5 ml min^{-1} then further washed with distilled water at 7 ml min^{-1} for 135 s. The cartridge was then washed with acetonitrile–water (10:90, v/v) for 35 s at 3 ml min^{-1}. After 5.25 min, the compounds were eluted directly onto the analytical column using the mobile phase at a rate of 1 ml min^{-1} for 45 s. A fresh extraction cartridge was used for each plasma sample. The extraction and analytical run times were synchronized in order to process one sample every 8 min, including extraction as well as analysis.

LC-MS/MS was performed on a Sciex (Thornhill, Ont., Canada) model API III triple quadrupole mass spectrometer, equipped with an upgraded collision cell and interfaced via a Sciex heated nebulizer probe to a liquid chromatograph consisting of a Hewlett Packard 1050 quaternary pump and autoinjector equipped with a vacuum degasser. A generic set of chromatographic conditions were used. A Zorbax C-18 RX column (25 cm × 4.6 mm i.d., 5 μm) supplied by MacMod (Chadds Ford, PA) was used. The mobile phase was 50% of Solvent A (acetonitrile–water–TFA, 90:10:0.1%) and 50% of Solvent B (methanol–water–TFA, 10:90:0.1%) at a flow rate of 1 ml min^{-1} to obtain an analytical run of less than 8 min for the 10 analytes plus the internal standard.

The nebulizer probe temperature setting was 500°C. The nebulizing gas pressure and auxiliary gas flow were set at 80 p.s.i. and 2 l min^{-1}, respectively. Chemical ionization was affected by a corona discharge needle operated at a voltage set to give a 4 mA current. The collision gas was argon at 270×10^{12} atoms cm^{-2}, the orifice potential was 45 V and the dwell time was 150 ms.

Quantitation was performed using selected reaction monitoring (SRM). The mass spectrometer was programmed to transmit the protonated molecules $[M + H]^+$ through the first quadrupole and following collision induced fragmentation in Q2, the appropriate (predominant) product ions were selected in Q3. The SRM chromatograms in Fig. 1 show the protonated molecular and product ion combinations used for quantitation of each substance. Peak area ratios of the analyte with respect to internal standard were computed using MacQuan version 1.3 software from Sciex. Calibration curves were constructed for each of the ten test substances using a weighed (reciprocal of concentration), linear regression of plasma concentration and the measured area ratio. Plasma concentrations of a given analyte in unknown samples were determined by interpolation from the appropriate standard curve.

2.5. Pharmacokinetic methods

Areas under the plasma concentration time curves (AUC) were calculated using the trapezoidal rule. $[AUC_0^\infty]$ was determined using the relationship:

$$[AUC]_0^\infty = [AUC]_0^t + \frac{C_t}{K} \quad (1)$$

where C_t is the plasma concentration at the last measurable timepoint and K is the elimination rate constant for the terminal phase. Plasma clear-

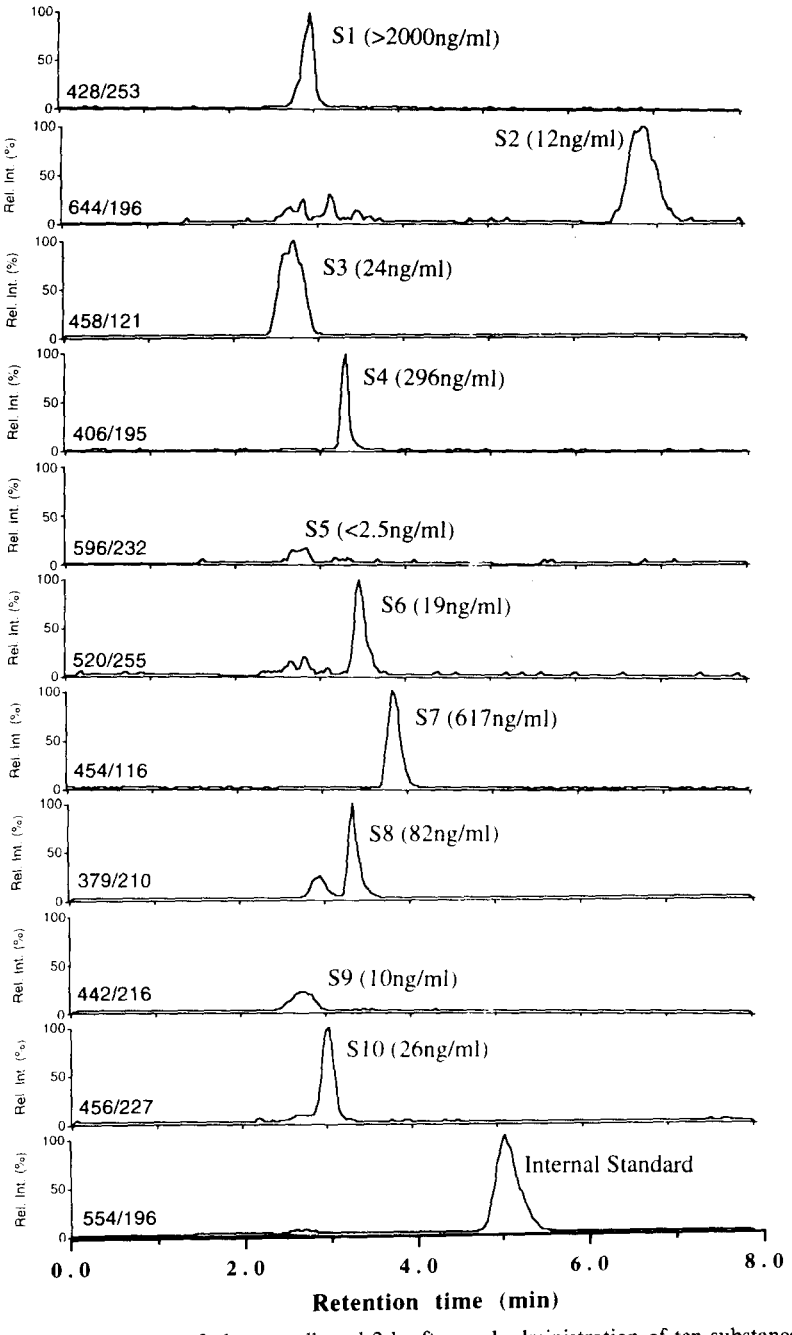

Fig. 1. SRM chromatograms of an extract of plasma collected 2 h after oral administration of ten substances simultaneously to a dog. The parent–product ion combinations and the measured plasma concentrations are indicated.

ance (Cl) was determined by dividing the intravenous doses by [$AUC0_0^\infty$]. The plasma half lives ($t_{1/2}$) were determined by log-linear least squares regression of the terminal phase of plasma concentration–time profiles. Bioavailability (F) was calculated as

Table 1
'Readback' values and calibration curve parameters for the simultaneous determination of test substances **1** through **10**

	Test substances identification number									
	Concentration (ng ml^{-1})	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mean curve readback values ($n = 2$)	2.5	1.9	2.4	2.4	2.0	1.8[a]	n.d[b]	2.0	n.d.[b]	2.3[a]	2.8
	5	6.2	5.2	5.0	6.2	5.9	4.1	5.0	4.7	5.2	5.6
	10	9.9	9.9	10.6	9.9	10.3	9.5	10.2	8.7	9.3	10.3
	50	46.9	49.3	48.4	52.2	54.1	53.7	53.9	52.4	51.1	52.1
	100	104	94.2	103	114	102.7	110	110	109	106	113
	200	197	208	194	210	209	210	205	209	196	209
	500	514	497	483	516	517	522	518	525	505	503
	1000	987	1005	1021	958	967	960	962	955	992	975
Slope		0.004	0.0140	0.0299	0.0040	0.0061	0.0043	0.0173	0.0120	0.0282	0.0121
Intercept		0.014	−0.0003	0.0018	0.0020	−0.001	0.0041	0.0271	0.0053	0.0092	0.0374
R		1.000	0.985	0.990	0.993	0.986	0.983	0.998	0.992	0.993	0.984
LOQ (ng ml^{-1})		2.5	2.5	2.5	2.5	5	5	2.5	5	5	2.5

[a] Value based on one determination.
[b] n.d., Not determined

Table 2
Extraction efficiency, accuracy and precision (mean% recovery ± %CV) of the assays for ten substances assayed simultaneously

Substance No.	Extraction efficiency (%)	Plasma concentration (ng ml^{-1}) of QC samples ($n = 6$)		
		5	50	500
1	86	102 ± 27	80 ± 13	77 ± 16
2	46	100 ± 19	101 ± 16	99 ± 14
3	97	121 ± 14	119 ± 18	108 ± 12
4	102	107 ± 10	115 ± 8	110 ± 9
5	106	119 ± 18	108 ± 12	108 ± 12
6	119	93 ± 18	108 ± 8	106 ± 12
7	88	93 ± 8	108 ± 7	109 ± 6
8	95	78 ± 19	112 ± 11	108 ± 9
9	96	90 ± 11	106 ± 6	101 ± 9
10	104	112 ± 21	99 ± 13	104 ± 9

$$F = \frac{AUC_{p.o.}}{AUC_{i.v.}} \times \frac{Dose_{i.v.}}{Dose_{p.o.}} \times 100 \quad (2)$$

where the subscripts p.o. and i.v. refer to oral and intravenous administration, respectively.

3. Results

The objective was to develop pharmacokinetic screens using the Prospekt on-line solid-phase extraction in conjunction with LC-MS/MS for the analysis of plasma from dogs receiving up to 10 test substances simultaneously. The selectivity and specificity of the LC-MS/MS and the structural similarity of the compounds facilitated the simultaneous extraction and chromatography of all 10 compounds. Representative chromatograms of plasma extracts from a dog dosed orally with the drug mixture are shown in Fig. 1.

3.1. Quality control

Rapid screening assays have evolved so that formal validation is no longer conducted prior to analysis of the test samples. Instead a limited validation is performed along with the unknown samples in a single analytical run. One generic extraction procedure was found which appears to work for most candidates within this class of compounds and the mobile phase and ionization conditions were slightly modified for each group of drug candidates. Two sets of spiked control plasma containing drug mixtures to serve as calibration standards along with independently prepared quality control samples at three concentrations of each substance ($n = 6$) were assayed along with the test samples. Calibration data for each substance are shown in Table 1 while the precision and accuracy of each of the 10 assays are shown in Table 2. The accuracy and precision of the 5, 50 and 500 ng ml^{-1} quality control samples were regarded as being within acceptable limits. The extraction efficiencies ranged from ca. 50 to 100% (Table 2).

3.2. Pharmacokinetics

Relevant pharmacokinetic parameters calculated from this experiment are shown in Table 3. Of principle interest are plasma half-life and oral bioavailability but our colleagues in Drug Discovery occasionally find use for plasma clearance and volumes of distribution. In this group, substances **1**, **4** and **7** were identified as the most promising candidates. Plasma concentration–time curves after oral administration of the ten substances to one of the dogs are shown in Fig. 2.

4. Discussion

There is a paradigm by which the evaluation of potential drug candidates is limited by the devel-

Table 3
Summary of the pharmacokinetics of 10 drug candidates following their administration as a mixture to dogs

Substance No.	C_{max} (p.o.) (ng ml^{-1})	Oral bioavailability (%)	$t_{1/2}$ (h)	Cl (ml min^{-1} kg^{-1})	V_D (l kg^{-1})
1	4230	117	1.9	2	0.2
2	59	n.d.	n.d.	n.d.	n.d.
3	112	29	1.9	24	1.1
4	1550	84	0.6	7	0.3
5	11	<1	0.8	18	0.6
6	133	n.d.	n.d.	n.d.	n.d.
7	1970	79	0.7	5	0.3
8	486	60	0.6	20	0.8
9	40	9	4.1	23	1.3
10	330	32	0.7	24	0.4

The oral and intravenous doses were 1 mg kg^{-1} and 0.5 mg kg^{-1} of each substance, respectively.
n.d., Not administered intravenously.

opment of suitable analytical methods for the determination of the substance in biological fluids. The use of reverse-phase HPLC has proved almost universal for the separation of most pharmaceutical candidates but this procedure, using conventional detection techniques, ultraviolet, fluorescence or electrochemistry, can struggle due to lack of specificity at low analyte concentrations. The development of API-MS/MS detectors has finally provided the high detection specificity that HPLC deserved.

HPLC with API-LC-MS/MS detection is now the foremost bioanalytical technique used by most pharmaceutical companies. The specificity afforded by this technique enables reliable methods to be developed in days rather than weeks. One powerful aspect of LC-MS/MS is that analytes which cannot be resolved chromatographically can still be specifically distinguished by their parent–product ion combinations. Thus, stable- and radio-isotopes can be separately determined [1] as of course may be mixtures of analogues. We have cautiously and gradually increased the number of analytes which may be accurately quantitated while maintaining adequate quality control. The latter is vitally important, although these assays are only required as pharmacokinetic screens, assay validation and quality control is still needed, since erroneous data from an unproven or unreliable assay could seriously misdirect the research effort.

In the experiment described here the extraction procedure was postulated from prior work but the total of 32 test samples processed were accompanied by two separate sets of calibration samples ($n = 10$) plus quality control samples ($n = 6$) at three concentrations. The LOQ of the assay for each substance was defined from the calibration and quality control results. Assays for multiple analytes may be used in different ways. Firstly, a single animal can be dosed with a mixture of the test substances. In such experiments the doses of the individual substances are reduced so that total drug load does not exceed that of a single candidate given alone. Such experiments are highly economic in both use of animals (e.g. 1 rather than 10) and also in the number of samples requiring to be prepared and assayed (e.g. 10 rather than 100). There are caveats however. The animal selected may absorb or metabolize the substances to an extent different from another animal. Additionally one particular candidate may inhibit metabolism of the others. The probability of such an event is reduced by the dose reduction but there is still the potential for one of the test substance mixture, being a particularly potent enzyme inhibitor, perturbing the metabolism of the others. To detect such effects we have included one particular compound whose pharmacokinetics have been evaluated both alone and in mixtures with each new batch of substances tested. Derived pharmacokinetic parameters for new substances can be evaluated with reference to those of this biological 'internal standard'.

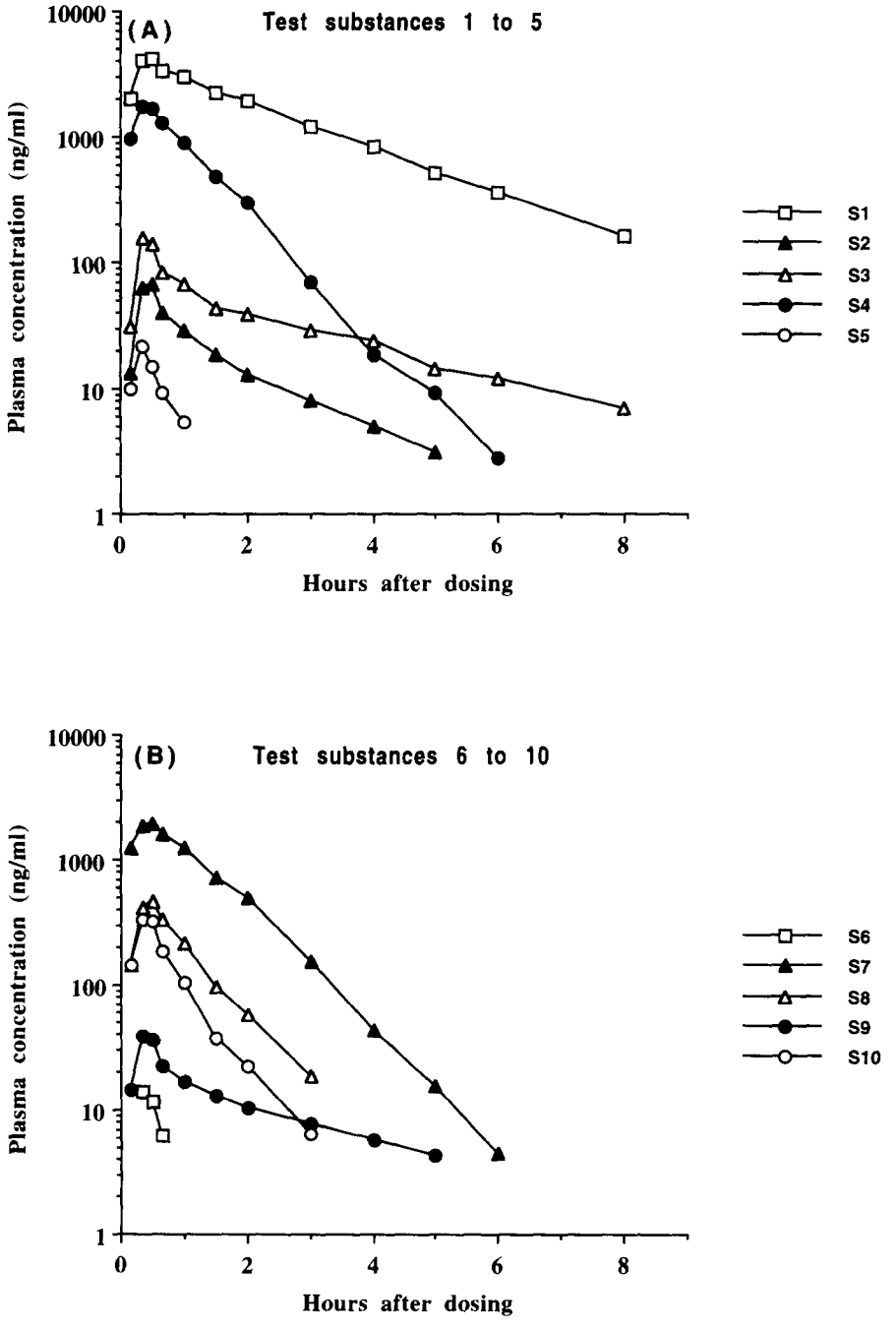

Fig. 2. Plasma concentration–time curves of 10 substances given orally as a mixture to one dog. The dose of each substance was 1 mg kg^{-1}.

An alternative dosing regimen involves dosing individual animals with a single substance with blood sample collection at a series of specified time-points. The assay is executed after plasma samples from say 10 animals are pooled at each time-point prior to analysis. Thus, analysis of ten

pooled plasma samples at 10 time-points yields the same amount of information as assaying 100 samples from the individual animals individually. There is, however, a sensitivity trade-off. If the assay is initially configured to detect say 0.5 ng ml^{-1} using 1.0 ml of plasma, the effective LOQ, when 10×0.1 ml aliquots are pooled, is 5 ng ml^{-1} per aliquot.

Fortunately, a detection limit of 5–50 ng ml^{-1} is generally adequate for drug screening. Should a substance be selected for further pharmacokinetic studies, an individual assay will be established and properly validated with an appropriately lower LOQ. It needs to be emphasized that the experiments described here are only meant to afford pharmacokinetic screening. Our objectives using such screening are to eliminate losers while neutrals and winners may be further evaluated. It is reassuring that screening such as described here have indeed resulted in the selection of a few promising candidates based on their observed pharmacokinetics. Perhaps, the paradigm of bioanalytical method development being a rate-determining step in drug candidate selection will soon no longer be appropriate.

Acknowledgements

The authors thank I-Wu Chen for preparing dosing solutions, dosing dogs and harvesting plasma, Dr. Samuel Graham for providing test substances and Maureen Hetzel for assistance in preparing this manuscript.

References

[1] J.D. Gilbert, T.V. Olah and D.A. McLoughlin, ACS Symp. Ser., 619 (1996) 330–350.
[2] D.A. McLoughlin, T.V. Olah, J.D. Ellis, J.D. Gilbert and R.A. Halpin, J. Chromatogr. A., 726 (1996) 115–124.
[3] B. Kaye, W.J. Herron, P.V. Macrae et al., Anal. Chem., 68 (1996) 1658–1660.
[4] S.D. Studenberg, J.D. Long, J.H. Woolf, C.J. Bruner, D. Wilson, and J.L. Woolley, J. Pharm. Biomed. Anal., 13 (1995) 1383–1393.
[5] H.G. Fouda, T.M. Twomey, and R.P. Schneider, J. Chromatogr. Sci., 26 (1988) 570–573.
[6] W.M. Mück and J.D. Henion, Biomed. Environ. Mass Spectrom., 19 (1990) 37–51.
[7] D. Barrón, J. Barbosa, J.A. Pascual and J. Segura, J. Mass Spectrom., 31 (1996) 309–319.