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Determination of the chlorofluorocarbon substitute 1,1,1,2-tetrafluoroethane (HFA-134a) in human and animal blood using gas chromatography with headspace analysis

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Abstract

A gas chromatographic procedure with headspace analysis and flame-ionization detection is described for the determination of the chlorofluorocarbon substitute 1,1,1,2-tetrafluoroethane (HFA-134a). A 0.5–2 ml sample of heparinized whole blood from a laboratory animal or human is added directly into a presealed headspace vial from which an equivalent volume of air has been removed. The internal standard 1,1,2,2-tetrafluoroethane is added and the sample frozen until analysis. Chromatographic separation is achieved using a PoraPlot Q porous-layer capillary column. The analytical range is 5.8–3298 ng/ml when 2-ml human blood samples are used. The concentration range of the calibration curve can be easily adapted to accommodate the concentrations expected in either pharmacokinetic or toxicokinetic studies. Automation of the assay permits the maximum number of samples to be processed in a day.

1. Introduction

Chlorofluorocarbons (CFCs) currently serve an important role as propellants in pharmaceutical metered-dose inhalers. These inhalers are used to deliver medicaments to the lungs, often for life-threatening conditions. During the 1970's, a link between active free radicals, such as chlorine atoms in CFCs, and the depletion of the stratospheric ozone was established [1]. In 1987, the United Nations adopted the Montreal Protocol to cause a reduction in, and the eventual elimination of, CFC production by the year 2000 in the 27 industrialized nations that signed the treaty [2]. Over 80 nations have now signed the Protocol, which has been revised to eliminate CFC production by 1996 [2].

In response to this environmental concern, the pharmaceutical industry is developing new propellants for pressurized metered dose inhalers that do not use CFCs [3]. Finding new suitable propellants that do not affect the ozone layer and have similar safety profiles to that of the current CFC propellants has not been an easy challenge [4]. One potential replacement propellant for CFCs is 1,1,1,2-tetrafluoroethane (HFA-134a). This chemical is a suitable propellant because it is a gas at room temperature and boils at −26.5°C [4]. Since HFA-134a contains no chlorine, it has been assumed to have essentially no ozone-depleting potential [5,6].

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Recent experiments have confirmed this assumption [7].

3M Pharmaceuticals is developing inhalation aerosols of salbutamol sulfate and beclomethasone dipropionate in a proprietary HFA-134a CFC-free system. These products are intended to be alternatives for the current CFC-containing metered-dose inhalers of these drugs. Before HFA-134a can be used in pharmaceutical products, however, it must undergo extensive testing for safety in laboratory animals and humans. The pharmaceutical development program for HFA-134a included measuring blood levels of the compound in the mouse, rat, rabbit, dog, and man [8], which necessitated the development of a bioanalytical method for HFA-134a. These blood level profiles were needed to document proof of absorption in toxicokinetic studies and for the calculation of pharmacokinetic parameters in laboratory animal and human metabolic studies. However, developing a bioanalytical method for a colorless and odorless gas that, based on current knowledge of CFC propellants [9,10] and radioactive studies with HFA-134a [11], was expected to occur at ng/ml concentrations in humans and animals, was a difficult challenge.

Previous studies have used radioactive techniques to monitor HFA-134a disposition in animals. One study measured HFA-134a in the bodies of rats using ^{14}C-radiolabelled HFA-134a [11]. Although that study provided extensive tissue distribution data of residual radioactivity, it could not provide the needed kinetic data for blood levels of the volatile parent compound, HFA-134a. Other studies utilized either ^{18}F-labelled HFA-134a for positron emission tomography (isotope half-life of 109 min) [12] or unlabelled HFA-134a for ^{19}F NMR (stable isotope) (personal communication). These fluorine monitoring techniques allowed the measurement of volatile HFA-134a in the bodies of animals; however, the dependence of these techniques on large, uncommon pieces of equipment with limited capacity did not allow these techniques to be viable for routine pharmacokinetic or toxicokinetic studies. The ^{18}F technique was further limited by the short half-life of the isotope. Other investigators have tried to measure the metabolism of HFA-134a in vitro by measuring the loss of fluorine with an electrode [13].

To our knowledge, this publication is the first report of a non-radioactive bioanalytical method for HFA-134a that is suitable for routine pharmacokinetics analyses. The method is a gas chromatographic procedure with headspace analysis and flame-ionization detection. Headspace gas chromatography has been widely used in a variety of analytical methods, ranging from the analysis of polymers to the measurement of ethanol levels in blood [14–16]. Headspace gas chromatography is particularly suited to the analysis of gases because most gases readily enter the headspace and there is a minimum amount of sample handling required, resulting in greater recovery of the gas from the sample.

The literature contains several examples of the application of headspace gas chromatography to the measurement of volatile anesthetics and CFC propellants [17–23] as well as a description of the gas chromatographic assay used to determine the purity of HFA-134a by one of the major producers of this gas [24]. None of the currently available headspace methods utilizes automated sample introduction or capillary columns, and none is specifically designed to detect HFA-134a. Also, because clinical and toxicological studies are commonly done at remote sites which require samples to be shipped to the analytical laboratory, we had to develop sample handling procedures that would make it possible to store samples prior to analysis. The current publication summarizes our efforts to improve upon past headspace gas chromatographic methods in developing a simple bioanalytical method for HFA-134a that would be suitable for pharmacokinetic studies.

2. Experimental

2.1. Chemicals

Pharmaceutical grade HFA-134a was purchased from Du Pont (Wilmington, DE, USA) and specified to be $>99.98\%$ pure. The internal

standard 1,1,2,2-tetrafluoroethane (HFA-134) was purchased from PCR (Gainesville, FL, USA) and was 99% pure. All other halocarbons came from either PCR or Aldrich (Milwaukee, WI, USA) and were the highest purity available. Trifluoroacetaldehyde was obtained from Lancaster Synthesis (Windham, NH, USA) and trifluoroacetic acid and 2,2,2-trifluoroethanol came from Aldrich. Sodium heparin solutions were purchased from Elkinns-Sinn (Cherry Hill, NJ, USA). Helium was >99.9999% pure (chromatographic grade). Nitrogen, hydrogen, and air were >99.99% pure (zero grade).

2.2. Materials

Teflon gas sampling bags and gas dilution bulbs were purchased from Alltech Associates (Deerfield, IL, USA). Pressure-Lok Series A-2 gas-tight syringes and side-port needles came from Dynatech Precision Sampling (Baton Rouge, LA, USA). The 9-ml headspace vials and butyl rubber septa (without Teflon) were obtained from Tekmar (Cincinnati, OH, USA).

2.3. Instrumentation conditions

A Tekmar 7000 headspace autosampler (with the 50-position 7050 carousel for increased sample capacity) was connected to a Hewlett-Packard (Avondale, PA, USA) 5880A GC using a 1/16 in. stainless steel union to join the headspace sampler transfer line with the GC carrier gas line just before the inlet [25]. Carrier gas flow was controlled by an external back-pressure regulator; injections were made with the GC septum purge and split vents capped off to prevent loss of sample. Conditions for the headspace sampler are given in Table 1.

A Chrompack (Raritan, NJ, USA) PoraPlot Q column (25 m × 0.53 mm I.D., with a 20 μm film thickness) was used with a helium carrier flow-rate of 6 ml/min. The GC inlet and initial oven temperatures were 100 and 35°C, respectively. After an initial time of 0.5 min, the oven temperature was increased at a rate of 13°C/min. Peaks were detected using a flame-ionization detector (FID) at 300°C, with air, hydrogen and nitrogen make-up gas flows of 380, 30, and 30 ml/min, respectively. Peak height was measured using a Hewlett-Packard integrator or a Waters 860 data system (Milford, MA, USA).

Table 1
Headspace sampler conditions

Platen temperature	60°C
Platen equilibrate time	0.50 min
Sample equilibrate time	8.0 min
Mixer	OFF
Pressurize time	0.10 min
Pressurize equilibrate time	0.25 min
Loop fill time	0.05 min
Loop equilibrate time	0.20 min
Inject time	0.50 min
Valve temperature	100°C
Transfer line temperature	100°C
Sample loop	500 μl
Pressurization setting	18 psi
Vial needle flow-rate	25 ml/min

2.4. Preparation of standards

Separate 50-ml gas sampling bags were filled with HFA-134a and internal standard. Each gas bag was evacuated using a vacuum source, filled with gas and evacuated again. This process was repeated two more times to ensure good sampling. Dilutions were made by removing a measured volume of gas from the gas bag using a gas-tight syringe with a sideport needle and adding it to a gas dilution bulb of known volume (measured gravimetrically by filling with water). Eight dilutions of HFA-134a (approximately 1:13 000 to 1:20) were prepared using gas bulbs ranging in nominal size from 125 to 1000 ml. A single dilution of internal standard was prepared by adding 1 ml of gas to a gas dilution bulb with a nominal volume of 125 ml.

Aliquots (2 ml) of blank heparinized whole human blood were sealed in headspace vials. Each vial was then spiked with 40 μl of a HFA-134a dilution and 40 μl of the internal standard dilution. Gases were added using gas-tight syringes with side-port needles by placing the needle through the septum and down into the blood. Each calibration vial was frozen on dry ice for 10 min to lyse the blood cells and create a

homogeneous liquid phase. Headspace vials were thawed at room temperature and shaken at 37°C for 30 min before being placed in the autosampler to ensure equilibration of the added gases with the blood. The final concentration of the calibration standards covered the range of 5.8 to 3298 ng/ml blood.

For toxicokinetic studies, calibration curves covering higher concentration ranges were prepared using either 0.5 ml of rat or 1.0 ml of dog blank heparanized blood. Assay modifications used for each concentration range are given in Table 2.

2.5. Preparation of biological samples

Two healthy male human volunteers (aged 26 and 28 years, and within 15% of ideal body weight) received two inhalations of 25 μl from a metered-dose inhaler containing salbutamol sulfate in the HFA-134a CFC-free system. Both subjects gave informed consent and the protocol was reviewed and approved by an independent institutional review board. Each inhalation was held in the lungs for 10 s, with a 30-s interval separating the two inhalations. Immediately prior to dosing, and at 2, 8, and 16 min after the start of the first inhalation, a 2-ml blood sample was drawn into a pre-heparinized plastic syringe and added directly to a pre-sealed headspace vial from which an equivalent volume of air had been removed. A 40-μl aliquot of the internal standard dilution was added and the sample frozen on dry ice. The sample remained frozen until analyzed as previously described. The exact volume of blood in each headspace vial was determined by weighing the vial before and after filling and dividing by the density of blood.

In a small laboratory experiment designed to show the suitability of the assay for toxicokinetic studies, an adult male Sprague–Dawley rat received a whole-body exposure to a 15% atmosphere (150 000 ppm) of HFA-134a for 1 h. Blood samples (0.5 ml) were drawn from the tail vein and handled as previously described.

2.6. Data analysis

For the calibration curve, HFA-134a/internal standard peak-height ratios were plotted against concentration. Weighted least-squares linear regression was used to calculate the slope, intercept, and coefficient of determination (r^2) using a weighting factor of 1/concentration [26].

2.7. Gas distribution coefficients

The distribution coefficient, K, for the partition of HFA-134a between the liquid and gas phases, was defined as:

$$K = C_L / C_G$$

where, C_L is the concentration in the liquid phase and C_G is the concentration in the gas phase.

The distribution coefficients for HFA-134a and the internal standard at 60°C were measured using spiked blood samples and the variable loading method described by Markelov [27].

Table 2
Assay conditions for calibration curves in three different species

	Human	Rat	Dog
Concentration range	5.8–3298 ng/ml	4.7–520 μg/ml	2.5–250 μg/ml
Sample volume (ml)	2.0	0.5	1.0
Sample loop volume (μl)	500	100	100
Internal standard concentration	555 ng/ml	320[a] μg/ml	160[a] μg/ml
Injector split ratio	Splitless	1:7	1:7

[a] Internal standard was used undiluted.

The amount of gas in the headspace, A_G, was calculated using equation:

$$A_G = \frac{A_T V_G}{(KV_L + V_G)}$$

where, A_T is the total amount of gas added to the headspace vial, V_G is the headspace volume and V_L is the liquid volume.

3. Results

3.1. Chromatography

Representative chromatograms of a blank blood sample and a 140 ng/ml HFA-134a calibration standard are shown in Fig. 1. Typical retention times for HFA-134a and the internal standard were 5.6 and 6.1 min, respectively. These two peaks were baseline resolved, with only minor peak tailing due to the size of the sample loop. No endogenous interference was seen in any of the blank blood (laboratory animal and human) samples tested.

The relative retentions of 14 compounds tested in this chromatography system are shown in Table 3. Trifluoroacetic acid, 2,2,2-trifluoroacetaldehyde and 2,2,2-trifluoroethanol have been suggested to be potential metabolites of HFA-134a [8,9]. These compounds were found to elute much later than either HFA-134a or the internal standard. Compounds 3–6 in Table 3

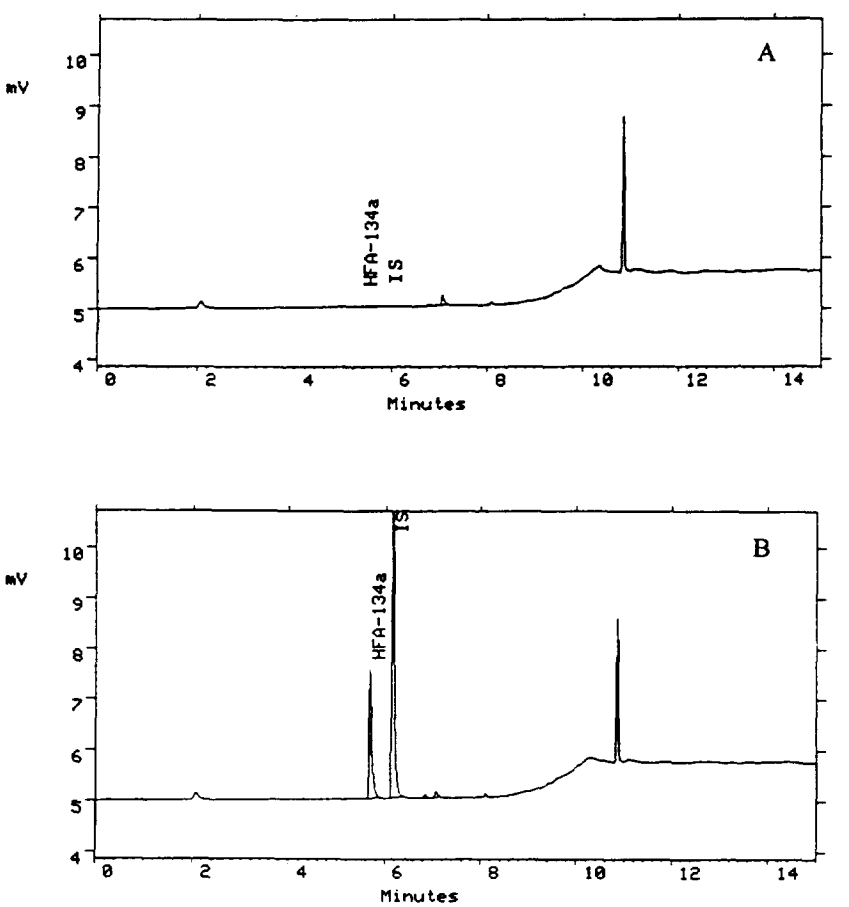

Fig. 1. Typical chromatograms of 2-ml whole human blood: (A) blank blood, (B) blank blood spiked with HFA-134a (140 ng/ml) and I.S., internal standard (555 ng/ml).

Table 3
Relative retention of selected compounds on the Poraplot Q column

	Compound	R_R[a]
1	1,1,2,2-Tetrafluoroethane (HFA-134)	1.15
2	1,1,1,2-Tetrafluoroethane (HFA-134a)	1.00
3	1,1,2-Trifluoroethane	1.31
4	1,1,1-Trifluoroethane	0.74
5	1,1-Difluoroethane	1.03
6	Fluoroethane	1.00
7	Trichlorofluoromethane (FC-11)	2.18
8	Dichlorodifluoromethane (FC-12)	1.32
9	1,2-Dichlorotetrafluoroethane (FC-114)	1.86
10	Ethanol	2.00[b]
11	Isopropanol	2.65[b]
12	Trifluoroacetic acid	≥4.0
13	2,2,2-Trifluoroethanol	2.16
14	Trifluoroacetaldehyde	2.05

[a] R_R = relative retention with respect to HFA-134a.
[b] These compounds continue to elute in the next run.

were tested as potential internal standards but none were as suitable as HFA-134 with respect to retention time, response, and peak shape. 1,1-Difluoroethane was separated from HFA-134a by only 0.1 min, but this separation was sufficient to clearly distinguish the peaks from each other. Only one compound, monofluoroethane, was found to co-elute with HFA-134a. Because monofluoroethane is not predicted to be a metabolic product of HFA-134a in mammals [11–13], and because the HFA-134a used in experiments contains at most only trace levels of this fluorocarbon [28], this co-elution should not pose a problem. Compounds 7–9 in Table 3 are CFCs currently used as propellants in metered-dose inhalers and all were well separated from HFA-134a.

3.2. Distribution coefficients

Under the conditions used for this assay, the distribution coefficients for HFA-134a and the internal standard were found to be 0.9 and 1.0. Following adjustment for the volumes of the liquid and gas phases in the headspace vial, it was calculated that 80% of the HFA-134a present in the vial and 79% of the internal standard were in the gas phase at 60°C when a 2-ml blood sample was used.

Table 4
HFA-134a/internal standard peak-height ratios for calibration standards prepared in 2-ml whole human blood from six different individuals, run on different weeks over a 3.5-month period

Concentration (ng/ml)	HFA-134a/internal standard peak-height ratio	
	Mean	R.S.D. (%)
5.8	0.01504	5.8
14	0.03727	2.5
38	0.09250	3.5
143	0.3505	3.0
294	0.7289	4.9
710	1.692	2.7
1480	3.599	1.0
3298	7.934	4.3

3.3. Calibration curve

The human assay was linear over the concentration range 5.8–3298 ng/ml with a signal-to-noise ratio of 19:1 at the limit of quantitation. Table 4 lists the peak-height ratios of HFA-134a and the internal standard from calibration curves

Table 5
Precision and accuracy data ($n = 5$)

Standard concentration (ng/ml)	R.E.[a] (%)	R.S.D. (%)
Within-day		
5.8	−5.2	14.7
14	+7.1	0.0
38	+0.5	1.2
143	+3.1	2.3
294	+2.7	5.8
710	+1.0	2.4
1480	+1.8	0.7
3298	−1.4	1.3
Between-day		
10	+8.6	10.0
294	+7.8	6.0
2200	+1.8	3.6

[a] Relative error = (found − added)/added.

Table 6
Storage stability data at −70°C for samples with demonstrated leakage

Storage time (days)	Nominal concentration (ng/ml)	Found concentration (ng/ml)	Internal standard[a] (%)
6	98	103	72
10	25	28	79
14	493	523	76
21	493	507	73

[a] Relative to the internal standard peak heigth of calibration standards prepared on the day of analysis.

prepared in six different blank human blood samples. The peak-height ratios were found to be reproducible over a 3.5-month period and in six different blood samples. The average slope and intercept of the calibration line were 0.0024 ± 0.0001 (mean ± S.D.) and 0.0020 ± 0.0013, respectively. The average r^2 value of the calibration linear regression was 0.9994 ± 0.005.

Within-day and between-day assay precision and accuracy data are presented in Table 5. The relative error at each concentration in the calibration curve was less than 10%. The coefficient of variation was 10% or less except at the limit of quantitation, where the within-day coefficient of variation was 14.7%.

Calibration curves were also prepared using 0.5-ml rat blood or 1-ml dog blood, and the assay modifications as described above for a higher concentration range. The rat calibration curve included the range 4.7–520 μg/ml, with $r^2 = 0.9999$, an intercept of −0.0057, and a slope of 0.0037. The dog calibration curve included the range 2.5–250 μg/ml, with $r^2 = 0.9999$, an intercept of 0.0003, and a slope of 0.0073.

3.4. Stability

Stability of samples was determined by spiking whole human blood with three concentrations of HFA-134a in headspace vials and storing the vials at either −20°C or −70°C for a period of up to 50 days. No significant change in concentration was seen over this period of time at either storage temperature and, on average, internal standard peak heights were within at least 90% of the expected value.

There were a few examples of vials that leaked during storage as evidenced by an internal standard peak height of 80% or less of that of the internal standard peak height in freshly prepared calibration standards. This occurred primarily in samples stored at −70°C and was not related to a defective seal on the vial. Since the leakage of HFA-134a and the internal standard almost certainly occurred at the same rate, leakage had no overall effect on the concentration data, and the observed concentrations correlated well with the amount of HFA-134a added (Table 6). We speculate that the septa became too stiff to seal well at −70°C.

Repeated freezing and thawing of a similar set of vials (up to 3 cycles) and storage at room temperature (up to 3 days) also did not change sample concentrations.

3.5. Biological results

Table 7 summarizes the blood concentrations of HFA-134a that were measured in the two volunteers given two inhalations from a metered-dose inhaler. The levels that were detected at 20

Table 7
HFA-134a blood levels in humans following two inhalations of HFA-134a salbutamol sulfate from a metered-dose inhaler

Time (min)	HFA-134a level (ng/ml)	
	Subject 1	Subject 2
0	<5.8	<5.8
2	330	461
8	23	56
16	8.4	24

min were approaching the limit of assay sensitivity.

In the laboratory animal experiment, the blood concentration that was observed in the rat during at 57th min of a 1-h exposure was 109 μg/ml. As was the case in the human study, this level decreased rapidly once exposure was completed, to 37 μg/ml at 17.5 min.

4. Discussion

The PoraPlot Q column was chosen for this analysis because of its ability to retain HFA-134a and the internal standard without the use of cryo-cooling. With this type of column, retention times were consistent for each particular column being used but were found to vary slightly between columns. The chromatographic conditions (initial oven temperature and oven temperature ramp) were optimized for each column to separate HFA-134a and the internal standard by 0.5 min.

An initial oven temperature of 35°C was found to result in narrower peaks, greater peak heights and better separation of HFA-134a and the internal standard than when the initial temperature was 50°C. The headspace sampler equilibration time of 8 min at 60°C was found to maximize the amount of gas in headspace. FID was chosen because of its linearity, reliability, and stability, and because its sensitivity for HFA-134a was greater than that of an electron-capture detector. FID is also used by a major producer of this gas to determine purity from more than 20 possible contaminants [24].

The size of the sample loop controlled the amount of headspace injected into the GC and affected the sensitivity of the method. The 500-μl sample loop gave the desired sensitivity for pharmacokinetic analyses and good chromatographic separations. Larger sample loops were tested. These gave more sensitivity, but resulted in more peak tailing. Smaller sample loops, e.g. 100 μl, were used for the higher concentration ranges needed to support toxicokinetic studies with relatively high doses.

The HFA-134a used in these experiments was assayed to be >99.98% pure by the manufacturer, which was adequate for the chromatography experiments. The purity of the internal standard was 99%, which was also acceptable since no extraneous peaks that would interfere with the analysis were found. The other halocarbons included in Table 3 were of the highest purities available; most were analytical grade. These compounds were sufficiently pure to determine retention times.

HFA-134 was chosen as the internal standard because of its chemical and chromatographic similarities to HFA-134a and because of its similar distribution coefficient. One shortcoming of this internal standard is that currently it is difficult to obtain from a commercial source. The addition of this internal standard made it possible to correct for any losses during analysis or storage.

The ability to store gaseous samples for up to 7 weeks prior to analysis was largely due to the properties of the headspace vial and septum. The selected vials have a beveled top designed to make a tight seal with the septum. The butyl rubber septa proved to provide a better seal than Teflon-coated septa when the vials were stored frozen. Because absorption of HFA-134a and internal standard by the butyl rubber was not found to be a problem, Teflon-coated septa were not required for this analysis.

The storage stability experiments were designed with the assumption that −70°C would be the preferred storage temperature because HFA-134a is a liquid at that temperature. However, samples were found to be equally stable at −20°C and −70°C. In addition, −70°C proved not to be a suitable storage temperature because sample vials tended to crack when thawed and septum seals were more likely to leak at this temperature when compared with −20°C.

The limited blood sampling in the human and rat indicates that HFA-134a can be absorbed following inhalation, but that its residence time in the body is relatively short, in the order of minutes, not hours. This finding is in agreement with the pharmacokinetic data that have been observed for current CFC propellants in both animals and human [9,10,29,30].

The large differences in HFA-134a concentrations that were observed between the rat and human primarily reflect the difference between a typical two-inhalation dose from a metered-dose inhaler and a 1-h exposure of a toxicokinetic dose, rather than a species difference. Again, this finding has been observed with the current CFC propellants [29,30].

5. Conclusions

This paper reports an assay for HFA-134a in the blood of laboratory animals and humans. Advantages of the method include simplicity, storage stability, and a high degree of precision. Automation of the assay permits the maximum number of samples, approximately 50, to be processed in one day. The concentration range of the calibration curve can be easily adjusted to accommodate the concentrations expected in either pharmacokinetic or toxicokinetic studies.

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