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THERMAL STABILITY EVALUATION OF DOPING COMPOUNDS BEFORE GC-MS ANALYSIS BY DSC

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The Medical Commission of the International Olympic Committee forbids the use of anabolic androgenic steroids, β-agonists, stimulant and narcotic compounds to improve athletic performance. In this work, we evaluated the thermal stability of 17 compounds by the use of the DSC for their potential GC-MS analysis either under free form or under TMS derivative form. In DSC, esterified and unesterified anabolic steroids were characterized by a true melting peak, followed by a large exothermic peak at about 251–316°C due to oxidative degradation. They could be analysed by GC-MS mainly under TMS derivatives. Hydroxylated and unhydroxylated stimulant compounds (xanthines) seemed to be more stable at high temperature. As unhydroxylated xanthines were not silylated with BSTFA – TMCS, their GC analysis would be done under their free forms. TMS derivatisation of albuterol hemisulfate and codeine phosphate is preferable. In our conditions, to analyse by GC-MS all 17 doping compounds in the same GC-MS run, the optimal silylation temperature and best column initial temperature were determined at both 60°C.

Keywords: *doping compounds, DSC, GC-MS, thermal stability, TMS derivatives*

Introduction

As part of the programme of drug test, the qualitative and quantitative study of doping compounds is very often realized by gas chromatography and mass spectrometry (GC-MS) [1–14]. Derivatization of compounds with polar groups is done in order to decrease their polarity, to increase their volatility, to improve their resolution in GC-MS and to stabilize thermolabile compounds. The most often reaction used is trimethylsilylation. Various reagents are used alone or in combination with catalysts. This reaction permits the derivation of compounds unstable in time; it could product secondary products leading the chromatographic analysis more complex and less specific [15–18]. So, in some cases, it is advised to study no derivatized compounds in order to avoid the development of multiple derivatives [19]. Sometimes, compounds without mobile-H cannot be sylated (e.g. caffeine).

The aim of this paper is to show the thermal stability by the use of DSC of 17 doping compounds (esterified and unesterified anabolic steroids, hydroxylated and unhydroxylated stimulants, β-agonist and narcotic) which are the most used unlawfully by sportsmen, and their previsional analysis by GC-MS, either under free form, or under TMS derivative form. Only qualitative sight was studied by GC-MS (presence or not of peak for each standard (free or TMS derivatized) and identification according to mass spectra).

Experimental

Chemicals and reagents

In Fig. 1 are the chemical structures of studied doping compounds.

Androsterone, nandrolone, nandrolone-17 propionate, stanolone-17 benzoate and albuterol hemisulfate were purchased from Riedel-deHaën (Germany); estradiol, etofylline, proxyphylline, caffeine anhydrous, racephedrine hydrochloride and theophylline anhydrous from Sigma (Germany); several compounds in conformity with the current European pharmacopoeia were given by pharmaceutical laboratories: testosterone propionate, norethindrone acetate, codeine phosphate anhydrous, fenfluramine hydrochloride and theobromine. BSTFA and TMCS were obtained from Riedel-deHaën (Germany); pyridine, methanol and hexane with higher purity grade from Merck (Germany).

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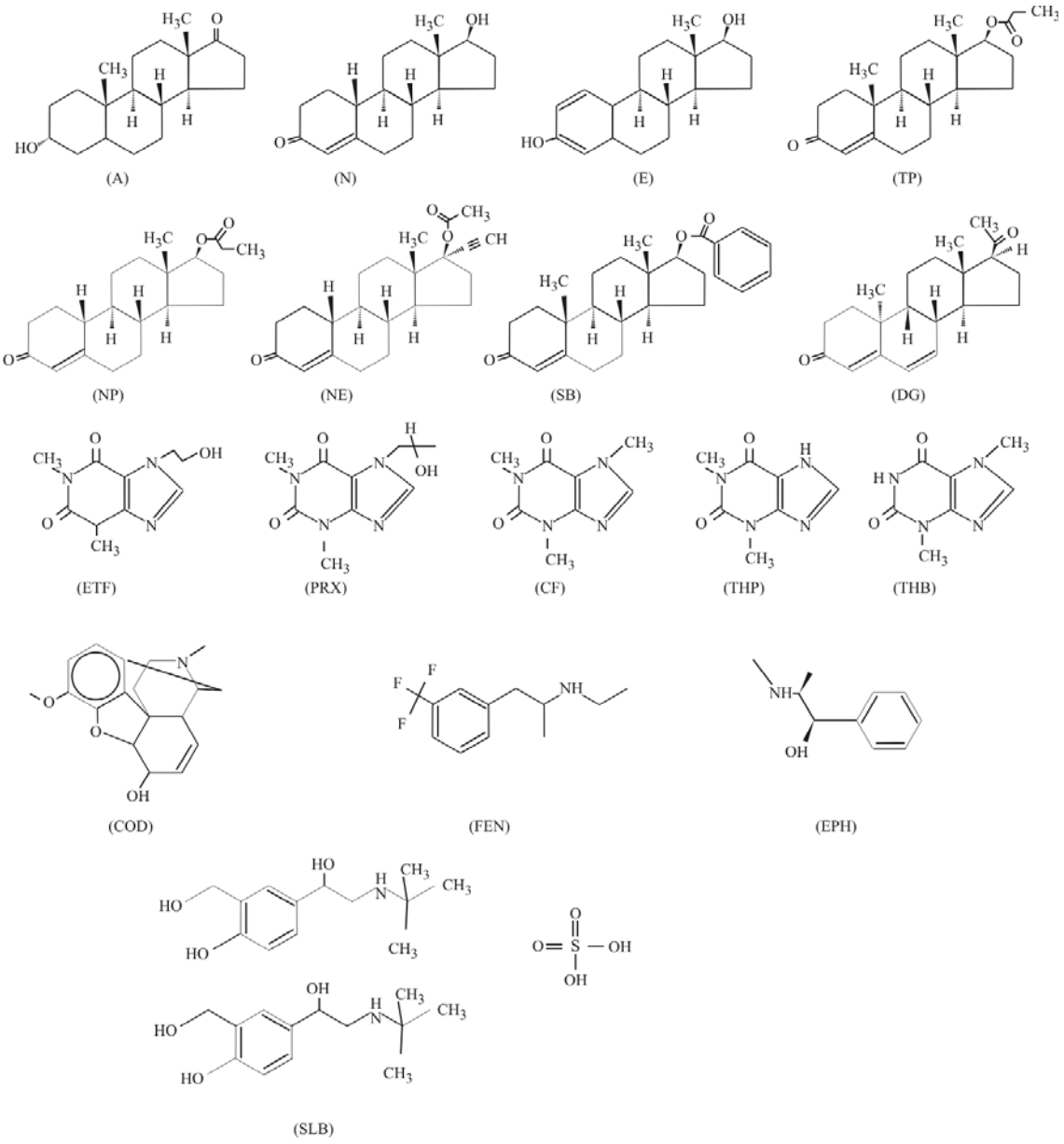

Fig. 1 Structure of the anabolic, stimulants, β-agonist and narcotic compounds considered in the GC–MS study: **(A)** androsterone, **(N)** nandrolone, **(E)** Estradiol, **(TP)** testosterone propionate, **(NP)** nandrolone-17 propionate, **(NE)** norethindrone acetate, **(SB)** stanolone-17 benzoate, **(DG)** dydrogesterone**, (ETF)** etofylline, **(PRX)** proxyphylline, **(CF)** caffeine, **(THP)** theophylline, **(THB)** theobromine, **(COD)** codeine, **(FEN)** fenfluramine, **(EPH)** racephedrine, **(SLB)** albuterol hemisulfate

Thermal analysis equipment and conditions

DSC Setaram 92 (Scientific and Industrial Equipment, Setaram S. A., 69300 – Caluire / France) was used, with 2°C min^{-1} heating rate, from room temperature to 570°C, under static air. The sample mass (about 10 mg) was put in aluminium crucibles. Pyrolyzed kaolin was used as thermally inert reference product. Temperature standardization was made by the melting of benzoic acid, indium, tin and lead. The indicated temperatures (°C) were attributed to the temperature of the maximum (T_{max}) or to the extrapolated temperature (T_{onset}) of each peak. In DSC, temperature accuracy was 0.1°C for melting point (T_{onset}) and 1°C for endothermic sublimation or exothermic peak (T_{max}). The phenomenon of melting was characterized after a first heating period until the apparition of an endothermic peak, followed by a second one of cooling with the apparition of an exothermic peak attributed to crystallization.

Chromatographic equipment and conditions

GC-MS analysis was performed with a Varian 3400 chromatograph interfaced to a MS Saturn 4D ion trap mass spectrometer (Varian SA, Les Ullis, France). It

Table 1 Temperature programming of the chromatographic column

GC used methods	Starting/°C	Hold/min	Rate/°C min^{-1}	End/°C	Hold/min
1	60	1	20	270	30
2	150	1	30	280	30
3	200	1	30	280	30
4	250	1	30	280	30

Four methods used in order to separate the 17 studied compounds (free form or TMS derivatized form)

was equipped with a DB5-MS capillary column (25 m x 0.25 mm i.d.; coating thickness, 1.0 μm). The temperature programming of the column will be optimized and displayed in Table 1. The carrier gas, helium, was supplied at 138 kPa head pressure. Sample injection volume was 1 μL (splitless mode). Injection port and detector were set at 250 and 285°C, respectively. The analyses were performed in the EI mode (ionisation energy = 70 eV). Three commercial libraries were used: WILEY®, NIST® and TR®.

Derivatisation conditions prior to GC-MS analysis

Stock solutions were prepared at a concentration of 1 mg mL^{-1} in methanol. Working solutions were prepared by diluting stock solutions. All solutions were stored at –20°C until analysis. Fifty μL of each solution were transferred in a 2 mL vial, methanol solvent was evaporated at 40°C under nitrogen. Two derivatisation protocols were tested:

- 300 μL of BSTFA-Pyridine (1:3 v/v), 60°C during 60 min, then evaporation under nitrogen and finally dissolution of the residue in 1 mL of hexane;
- 300 μL of BSTFA-TMCS (99:1 v/v), 60°C during 60 min, then evaporation under nitrogen and finally dissolution of the residue in 1 mL of hexane.

Samples were prepared once for each tested derivatization temperature and injected into GC-MS twice.

Results

Four groups of compounds were studied. The first group contains anabolic steroids: unesterified (nandrolone, estradiol, dydrogesterone, androsterone); esterified (nandrolone-17 propionate, testosterone propionate, norethindrone acetate, stanolone-17 benzoate). The second group contains xanthines (stimulant compounds): unhydroxylated (fenfluramine hydrochloride, caffeine anhydrous, theophylline anhydrous, theobromine); hydroxylated (proxyphylline, etofylline, racephedrine hydrochloride). The third and the fourth groups contain only one compound, albuterol hemisulfate and codeine phosphate anhydrous, respectively.

Thermal analysis

Main results are in Table 2 (N° CAS of each compound; theoretical melting temperature (°C); observed melting temperature (T_{onset})). DSC curves showed mainly a first endothermic peak attributed to melting, than an exothermic peak of oxidation (example of androsterone in Fig. 2) or an endothermic peak of sublimation (example of proxyphylline in Fig. 3).

GC-MS analysis

In Table 3 are the GC-MS results obtained on TMS derivatized and underivatized compounds. The 17 compounds are classified according to their retention time (RT). Molecular ion fragments are also indicated. Results were obtained according to method 1. TMS derivatized compounds were characterized according to their mass spectra (Table 4), they all were similar to those described in reference [23].

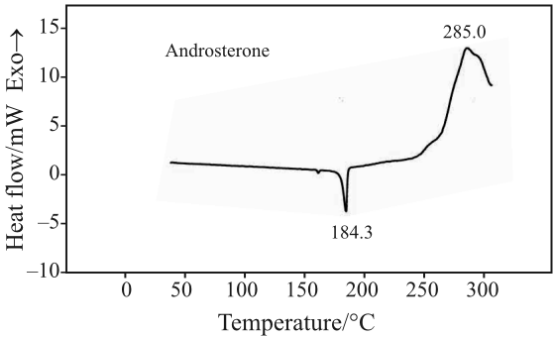

Fig. 2 DSC curve of androsterone

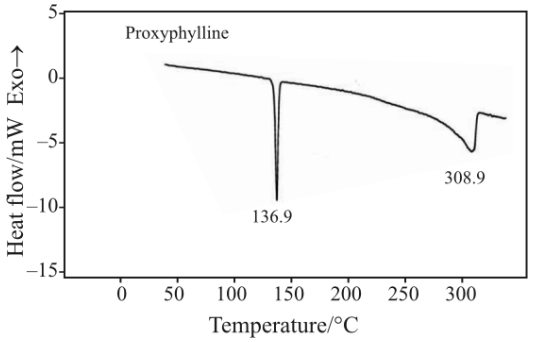

Fig. 3 DSC curve of proxyphylline

Table 2 DSC results

Studied compounds	N°CAS	Theoretical T_f/°C (*)	Observed T_f/°C T_{onset}	Exothermic peak T_{max}/°C
		Unesterified anabolic steroids		
Nandrolone	434–22–0	123–125	122.5	288
Estradiol	50–28–2	173–179	176.3	316
Dydrogesterone	152–62–5	169–170	167.8	303
Androsterone	53–41–8	181–185	179.7	285
		Esterified anabolic steroids		
Nandrolone-17 propionate	7207–92–3	55–60	41.8	312
Testosterone propionate	57–85–2	118–122	119.5	292
Norethindrone acetate	51-98-9	161–162	160.7	251
Stanolone-17 benzoate	1057-07-4	200–202	198.0	297
	N°CAS	Theoretical T_f/°C (*)	Observed T_f/°C T_{onset}	Endothermic peak of sublimation T_{max}/°C
		Hydroxylated stimulants		
Proxyphylline	603–00–9	135–136	134.3	309
Etofylline	519–37–9	158	160.1	313
Racephedrine hydrochloride	134–71–4	187–188	188.1	255
		Unhydroxylated stimulants (xanthines)		
Fenfluramine hydrochloride	16105–77–4	168–172	168.3	240
Caffeine anhydrous	52–08–02	238	234.2	275
Theophylline anhydrous	58–55–9	270–274	268.5	326
Theobromine	83–67–0	357	– (**)	332
	N°CAS	Theoretical T_f/°C (*)	Observed T_f/°C T_{onset}	Exothermic peak T_{max}/°C
		B-agonist and narcotic		
B-agonist: albuterol hemisulfate	51022–70–9	–	168.3 *D* T_{max}	292
Narcotic: Codeine phosphate anhydrous	52–28–8	227.5	177.7 *F* T_{onset} 225.1 *F* T_{onset}	266

T_f=melting temperature; *F*=Fusion; *D*=Decomposition. The indicated temperature (°C) of the presented molecules were attributed to the extrapolated (T_{onset}) of the endothermic melting peak (T_f), and to the maximum (T_{max}) of the exothermic peak and of the endothermic peak of sublimation or of the endothermic decomposition peak.(*) see references [20–22]; (**) No melting peak was observed, but sublimation endothermic peak

Discussion

The first group of anabolic steroids (unesterified and esterified) is characterized by a true melting peak (T_f), followed, at about 251–316°C, by a large exothermic peak due to the oxidative degradation of the molecules. Nandrolone-17 proprionate showed a broad endothermic melting peak (T_{onset}= 41.8°C; T_{max}= 58.3°C). This T_f decreasing (41.8 instead of 55–60°C) could be due to the presence of impurities.

Afterwards, we injected a mixture of anabolic steroids (unesterified and esterified) into GC-MS at different initial temperature (T_i) of column. At T_i=60°C (method 1) analysis duration was longer than that of 150°C (method 2). At T_i=200°C (method 3) and at T_i=250°C (method 4) we noted the absence of peak explained by a compound decomposition. This degradation was also observed by DSC.

The derivatization by trimethylsilylation increases volatility and stability of compounds. The same mixture silylated at 60°C and 115°C during one hour was injected at various T_i of GC-MS. We observed the separation of all compounds with a decreasing in the analysis duration, and also a decreasing in sensibility according

Table 3 Retention times and molecular ions observed on TMS derivatized and underivatized compounds with the use of GC-MS method 1

	Compounds(500 μg mL^{-1} in methanol)		Compounds-TMS(50 μg mL^{-1} in hexane)	
	RT/min	Molecular ion	*RT*/min	Molecular ion
Fenfluramine hydrochloride	7.38	232	–	–
Racephedrine hydrochloride	8.70	166	8.55*	238*
Theobromine	11.86	180	–	–
Caffeine anhydrous	11.90	194	–	–
Albuterol hemisulfate	–	–	12.66	456***
Theophylline anhydrous	12.95	180	–	–
Etofylline	14.06	224	13.56	369**
Proxyphylline	14.33	238	13.80	383**
Codeine phosphate anhydrous	18.70	299	19.16	371*
Androsterone	21.55	290	20.02	347*
Nandrolone	23.43	275	23.28	419**
Estradiol	24.63	272	24.05	416**
Norethindrone acetate	30.68	341	30.43	414*
Nandrolone -17 propionate	30.82	331	30.56	403*
Testosterone propionate	34.06	345	33.50	417*
Dydrogesterone	35.06	313	34.85	385*
Stanolone-17 benzoate	–	–	–	–

– not detected; * mono TMS; ** bis TMS; ***tris TMS

to the increasing of T_i. This decreasing is important for unesterified anabolic steroids at 115°C and 150°C for derivatization temperature and T_i, respectively. These compounds are instable and could decompose in these conditions of silylation and separation (Tables 5 and 6).

Esterified anabolic steroids showed a complex chromatogram with several peaks corresponding to partial derivatization. This procedure seemed to be not appropriate for these compounds. So, ulterior quantitation of these esterified anabolic steroids could be done under their non derivatized form. We could explain the non derivatization of stanolone-17 benzoate by a heavier molecular mass and an upper melting temperature.

The second group corresponding to xanthines (hydroxylated and unhydroxylated) gave a melting temperature corresponding to those given by references [20–22], or slightly lower. In opposite to the 1st group, here, the 1st melting endothermic peak was always followed by a sublimation endothermic peak with a maximum at 240 to 332°C, except for theobromine which showed only a sublimation endothermic peak with a T_{max} at 332°C. Xanthines seemed to be more stable at high temperature, in opposite to anabolic steroids, because any exothermic degradation peak was observed.

In our GC-MS analysis, these compounds had short retention times. Injection of silylated and unsilylated xanthines at high T_i (200°C and 250°C) did not show any peak in chromatograms, because their migration was practically similar to the solvent. Method 1 (T_i=60°C) was the best method for a good resolution.

In DSC, albuterol hemisulfate (β-agonist) decomposed easily at 168°C (T_{max} of the first endother-

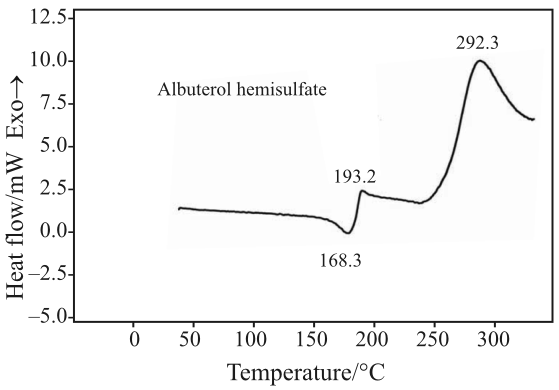

Fig. 4 DSC curve of albuterol hemisulfate

Table 4 GC-MS results: characterization of TMS derivatized compounds with the use of method 1

Compounds	MM	*m*/*z* fragments (abundance/%)								
Unesterified anabolic steroids										
		MM –15	MM –44	MM –73	MM –90	MM –73 –90	MM –90 –15	MM –129	MM –73 –44	MM –73 –44 –15
N-bis TMS	419 (28)			346 (60)	33 (30)	256 (100)				
E-bis TMS	416 (100)	401 (18)			326 (35)			285 (73)		
DG-TMS	385 (13)		341 (27)	312 (87)					268 (100)	253 (24)
A-TMS	362 (12)	347 (30)			272 (100)		257 (53)			
Esterified anabolic steroids										
			MM –73	MM –57	MM –73 –57	MM –90	MM –90 –57			
NP-TMS	403 (13)		331 (35)		274 (30)		256 (85)			
TP-TMS	417 (08)		344 (25)		283 (40)		267 (15)			
NE-TMS	414 (12)		341 (80)	355 (38)			282 (52)			
Hydroxylated stimulants										
			MM –73	MM –73 –15	MM –73 –73	MM –73 –73 –44	MM –73 –73 –57	MM –90	MM –90 –15	MM –90 –15 –16
PRX-bis TMS	383 (38)		310 (32)	295 (57)	237 (15)		180 (30)			
ETF-bis TMS	369 (10)		296 (45)	281 (57)	224 (15)	180 (100)				
EPH-TMS	238 (90)		165 (3)					148 (7)	133 (3)	117 (4)
B-agonist and narcotic										
			MM –15	MM –87	MM –87 –73	MM –28	MM –28 –31	MM –73	MM –90	
B-agonist: SLB tris-TMS	456 (45)		440 (10)	369 (100)	295 (3)					
Narcotic: COD-TMS	371 (100)		356 (10)			343 (20)	312 (15)	298 (12)	281 (20)	

m/*z* fragments: MM, 15, 16, 28, 31, 44, 57, 73, 87, 90, 129, correspond to: molecular mass, CH_3, O, CO, OCH_3 , CH_3CO, CH_3 CH_2CO, Si $(CH_3)_3$, CH_3 $NHC(CH_3)_3$, $HOSi(CH_3)_3$, A-ring respectively

mic peak) and then oxidized weakly at 193°C which corresponds to the exothermic peak T_{max}. This degradation could be explained by a possible dehydration of the alcoholic function (catalyzed by sulphuric acid present in the molecule) and then oxidation. A large exothermic peak was observed at 292°C (Fig. 4).

In GC-MS, the absence of peak could be explained by the same phenomenon, a possible dehydration, then carrying a possible reaction on itself to give molecular mass increasing and volatility decreasing.

In opposite, the TMS-compound was observed for albuterol hemisulfate. Three TMS groups react with the three OH groups of albuterol, and prevent a possible ulterior dehydration.

In the case of albuterol, T_i could have an influence on the derivatization process. We observed an increasing of the area ratio of T_i at 150°C over 60°C (45.5%) (Table 5). This increasing could be due to an additional derivatization in column.

Table 5 Ratio (%) between the areas of analytes as TMS derivatives obtained for derivatisation temperature of 115 over 60°C

Oven temperature T_i/°C	Derivatisation $T°$/°C	SLB tris-TMS	COD–TMS	A-TMS	N bis-TMS	E bis-TMS
60	115/60	73.9		–19	–83.6	–19.8
150	115/60	42.3		–117	–87.6	–89.1

Table 6 Ratio (%) between the areas of analytes as TMS derivatives obtained for T_i of 150°C over 60°C

Oven temperature T_i/°C	Derivatisation $T°$/°C	SLB tris-TMS	COD–TMS	A-TMS	N bis-TMS	E bis-TMS
150/60	60	45.5		5.9	–59.1	–9.7
	115	–20.5	33.01	–71.6	–62.59	–73.1

If we used TMCS instead of pyridine in the derivatization process, the obtained derivative is also albuterol-trisTMS. When the T_i increased from 150°C to 250°C, we observed:

- a decreasing in sensibility: peak area at 150°C, 200°C and 250°C were 1.7, 40 and 40 lower than 60°C respectively,
- an increasing of the broadness of the peak and a dividing into two. These two peaks had the same mass spectra. So, residual sulphuric acid present in the derivatized sample could lead to isomerization.

Codeine phosphate anhydrous (narcotic) undergoes to two melting peaks (Fig. 5). If the second one is surely attributed to the melting of the phosphate salt, the first one could correspond to a mixture of codeine base, released from the salt, and the salt itself. Only one peak appeared in chromatographic analysis after the injection of a codeine phosphate alcoholic solution.

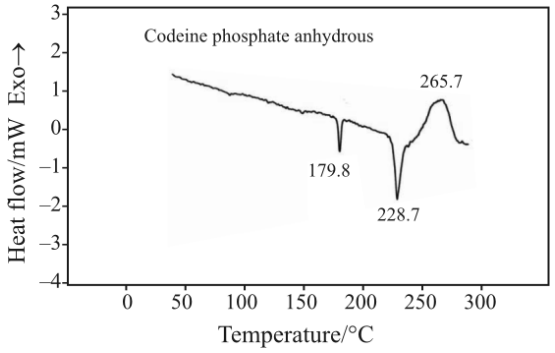

Fig. 5 DSC curve of codeine phosphate anhydrous

Increasing derivatization temperature favoured the trimethylsilyl reaction [24]. When derivatization temperature of codeine phosphate anhydrous was realized at 60°C, two peaks were observed corresponding to phosphate tetra-TMS and codeine under free form, respectively. In opposite at 115°C, the second peak is attributed to codeine-TMS.

Conclusions

In conclusion, several compounds, showing a low thermal stability (like for examples anabolic compounds) by DSC, could be analysed by GC-MS mainly under TMS derivative forms. In opposite, xanthines, showing a better thermal stability, could be analysed without TMS derivatisation. The situation of albuterol seemed to be more complex; degradation occurred in the presence of sulphuric acid, therefore TMS derivative is preferable. It is also preferable for codeine phosphate. Finally, in our conditions, to analyse by GC-MS all 17 doping compounds in the same run, the optimal silylation temperature and best column initial temperature were determined at both 60°C. In the last two years, few works were done on the thermal stability steroids [25].

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